uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Diana Malouf

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.A.Sc. (Chemical Engineering)

GRADE / DEGREE

Department of Chemical Engineering

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Mesenchymal Stromal Cell Differentiation into Osteoblasts on Modular Aritifical Proteins with Bioactive Ligands

TITRE DE LA THÈSE / TITLE OF THESIS

T. Matsuura

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

M. Griffith

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

X. Cao

C. Lan

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Mesenchymal Stromal Cell Differentiation into Osteoblasts on Modular Artificial Proteins with Bioactive Ligands

by

Diana Malouf

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirement for the degree of

Master of Applied Science

in

Department of Chemical and Biological Engineering

University of Ottawa

© Diana Malouf, Ottawa, Canada 2009

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-58184-1
Our file Notre référence
ISBN: 978-0-494-58184-1

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

Modular self-assembling *de novo* triblock proteins (designated CRC) that contain a central bioactive domain with associating ends allowing preferential formation bundles have been developed. These proteins subsequently self assemble into hydrogel networks. The modularity of these triblock systems allows for different combinations of end and centre domains to modulate cell differentiation and phenotypic changes. In this study, we examined the effect of CRC peptides with bioactive ligands from extracellular matrix proteins involved in differentiation and repair activities, fibronectin (RGDS) and laminin (LQVQ), on mesenchymal stromal cell (MSC) differentiation into osteoblasts.

Briefly, CRCs were covalently surface grafted onto collagen scaffolds via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) chemistry. Four peptide combinations tested were: P-CRC, P-CRC-RGDS/CRC, P-CRC-LQVQ/CRC and CRC-RGDS/CRC-LQVQ. They were examined for effects on proliferation and differentiation of MSCs in culture. No significant differences in proliferation rates were observed amongst samples. MSCs grown on CRC alone were unhealthy. All cells cultured on the peptide substrates, except for a combination of CRC-RGDS/CRC-LQVQ, had calcium deposits that were indicators of osteogenic differentiation. Cells grown on CRC/CRC-RGDS substrates showed most differentiation. The combination of RGDS/LQVQ peptides appeared to promote retention of an undifferentiated progenitor cell state.

We have therefore shown that modular, self-assembling peptides have the potential to be useful in modulating differentiation or retention of the stem cell phenotype. With further development, they could possibly be used to develop or enhance biomaterials scaffolds for use in modulating stem cell behaviour in potential therapeutic applications.

Résumé

Des peptides (nommer CRC) *de novo* en tribloques capable de s'associer ensemble pour former des hydrogels ont ete developpe. Ils contiennent aussi des domaines centraux à séquences bioactives. Leur association en mèche d'hydrogel est facilitée par leurs extrémités capables de s'associer en hélices hétérotrimères. De plus la variation des bloques centrales par le changement des séquences bioactives permet la combinaison de differents types de peptides pour avoir ulterieurement diverse effet sur la différentiation et les changements phénotypiques des cellules. Dans cette étude, nous avons examiné l'effet de peptides CRC, contenant des séquences bioactives provenant de protéines de la matrice extracellulaire qui sont connus pour leurs activités réparatrices et de différentiation des cellules tel que fibronectine (RGDS) et laminine (LQVQ), sur la différentiation en ostéoblaste des cellules souches du mésenchyme (MSC),

En bref les CRCs ont été chimiquement attachés sur les surfaces de matrices de collagène par l'utilisation d'un agent de liaison, l'1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Quatre combinaisons de peptides ont été étudiées: P-CRC, P-CRC-RGDS/CRC, P-CRC-LQVQ/CRC and CRC-RGDS/CRC-LQVQ. Ces combinaisons ont été examinées pour leurs effets sur la prolifération et la différentiation des MSC en culture. Aucune différence signifiante en terme de prolifération a été observe parmi tous les échantillons. Les MSCs cultivées sur des P-CRC n'étaient pas trop saines. En plus tous les échantillons sauf celui-ci contenant la combinaison CRC-RGDS/CRC-LQVQ contenaient des dépositions de calcium, un indicateur de la différentiation en ostéoblaste. Les cellules cultives sur la combinaison P-CRC/CRC-RGDS ont démontré le plus de différentiation. Par contre la combinaison des séquences RGDS/LQVQ a permis de retenir l'état non différentié progéniteurs des cellules souches.

Dans cette étude on a donc montré que les peptides CRCs sont utiles pour prédéterminer la différentiation ou pas des cellules souches. En les développant davantage, ces peptides pourraient être utilisés pour améliorer les matrices de biomatériaux afin de moduler le comportement de cellules souches dans des applications thérapeutiques importantes.

Acknowledgements

I would like to take this opportunity to thank all those who helped me in some way or many to accomplish this project.

First of all I wish to thank my Supervisors Dr. May Griffith and Dr. Takeshi Matsuura for their continued assistance and patience during this research and compilation of this study.

Also I wish to thank all those who collaborated in making this project happen, such as Dr James Harden and Dr. Stephen Fisher from the University of Ottawa for providing me with the CRC peptides and helping with the radioactive experiments, Dr Hai-Quan Mao from the Department of the Materials Science and Engineering at the John Hopkins University for allowing us to do all the radioactive experiments in his laboratory in Baltimore and Melissa Huynh for helping with the cell cultures.

I would also like to thank everyone else who helped me with this project in particular my colleagues in the Griffith lab such as Kim Merrett, Dr. Chao Deng, Dr. Fengfu Li, Dr. Jae-Il Ahn and others for their support, friendship and motivation.

Finally I wish to thank my family, friends and God, for supporting me throughout my Masters.

Table of Contents

List of Figures

reactive amine group (on either substrate collagen or CRC) attacks the unstable intermediate to form and amide bond and hence releasing the EDC to the surroundings (step3).

Figure 8: Chemical structure of the Sulfo EGS crosslinker [56].

Figure 9: Covalent grafting of CRC peptides to the surface of a collagen scaffold via Sulfo EGS. The different steps are highlighted by the numbers 1 and 2. Step 1 starts when the amine group on either CRC peptides or collagen scaffolds attacks the carbon atom from the ester group of the Sulfo EGS. This results in an amide bond between the collagen (or CRCs) and the sulfo EGS. The other end of the Sulfo EGS also reacts with another amine group (located on either collagen or CRC, step 2) to form another amide bond.

Figure 10: Remaining peptide on collagen scaffolds versus the initial peptide concentration in solution. P-CRC is compared to CRC-RGDS.

Figure 11: Percentage of peptide remaining on 10% collagen scaffolds at different crosslinker concentrations and type and after undergoing intense washing of gels after peptide grafting. P-CRC was evaluated in this study. In the case of Sulfo EGS, this crosslinker was dissolved in either water or PBS.

Figure 12: Amount of P-CRC peptide remaining on 10% collagen scaffolds at different EDC crosslinker concentration, with NHS activator and without it. Only P-CRC was evaluated in this study.

Figure 13: Images of MSCs cultured on 10% collagen scaffolds modified with CRC peptides and their controls. Pictures were taken on day 14 culture. Samples correspond to cells on P-CRC, P-CRC/CRC-LQVQ, P-CRC/CRC-RGDS, CRC-RGDS/CRC-LQVQ, Unmodified collagen scaffold, EDC modified collagen scaffold, Peptide only well (sample consisting of CRC-RGDS and CRC-LQVQ at an equal ratio) and Empty well. A 200X times magnification was used.

List of Tables

equality of loading abilities of both types of CRC peptides, the effect of concentrations of peptide in solution and the interaction between concentrations and type of peptide.

Table C7: Raw data for P-CRC stock solutions prepared for the experiment involving grafting of peptide with different crosslinkers and further washing in 10% SBS-PBS

Table C8: : Before and after washing with 10% SDS PBS raw data, for P-CRC grafting on 10% collagen scaffolds using different crosslinkers.

Table C9: Raw data used for statistical analysis of inequality of crosslinkers used when P-CRC was grafted on 10% collagen scaffolds and further washed with 10% SDS-PBS.

Table C10: Summary of percentage of P-CRC remaining on 10% collagen scaffolds following crosslinking with different chemicals. Data used in statistical analysis.

Table C11. One Way ANOVA results used for testing of inequality of crosslinkers used when P-CRC was grafted on 10% collagen scaffolds and further washed with 10% SDS-PBS (counts, sums, averages and variances).

Table C12: One Way ANOVA results obtained for testing of inequality of crosslinkers used when P-CRC was grafted on 10% collagen scaffolds and further washed with 10% SDS-PBS

Table C13: Raw data for P-CRC stock solutions prepared for the experiment involving grafting of peptide with EDC and NHS chemicals and further immersion in 10% FBS solutions

Table C14: Raw data for P-CRC grafting with EDC and NHS chemicals to 10% collagen scaffolds and further immersion in 10% FBS solution.

Table C15: Data corresponding to the amount of P-CRC peptide (ng) in each sample inmmersed in 10% FBS solutions. This data was used for statistical Analysis by Two Way ANOVA with replication (triplicates of each sample are shown for each crosslinker and at

every hour studied). Note that due to lack of data, the values for 1600uM-EDC were not taken into account in test.

Table C16: ANOVA: Two-Factor ANOVA with Replication of data corresponding to the amount of P-CRC peptide (ng) in each sample inmmersed in 10% FBS solutions.

Table C17: Summary of results for Two-Factor ANOVA with Replication of data corresponding to the amount of P-CRC peptide (ng) in each sample inmmersed in 10% FBS solutions.

Legend

MSC: Mesenchymal Stromal Cells.

CRC: Refers to all types of CRC peptides in general.

RGD: Arginine-Glycine-Aspartic Acid.

RGDS: Arginine-Glycine-Aspartic Acid-Serine.

LQVQ: Leucine- Glutamine- Valine-Glutamine.

IKVAV: Isoleucine- Lysine-Valine-Alanine-Valine.

P-CRC: Refers to CRC peptides that lack any bioactive sequence.

CRC-RGDS: Refers to a type of CRC peptide containing the RGDS bioactive sequence in its middle block.

CRC-LQVQ: Refers to a type of CRC peptide containing the LQVQ bioactive sequence in its middle block.

q: Amount of peptide adsorbed peptide.

C: Concentration of peptide in solution.

k_F: Adsorption constant that indicates the capacity of the substrate for adsorbing the peptide.

No X-linking: Refers to the samples where peptides were adsorbed/absorbed to the sample, *i.e.* no crosslinkers were used.

EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

NHS: *N*-hydroxysuccinimide

Sulfo EGS: Ethylene glycol bis [sulfosuccinimidylsuccinate]

C: P-CRC.

CL: P-CRC/CRC-LQVQ.

CR: P-CRC/CRC-RGDS.

LR: CRC-LQVQ/CRC-RGDS.

N: Unmodified collagen.

E: EDC modified collagen.

P: Peptides alone.

W: Tissue culture plate well.

Chapter 1: Introduction

When organs and tissues are lost due to damage from injury or diseases, the common response of doctors has always been to replace them by the use of donated organs or tissues with informed consent. However, there is a shortage of donor organs, which compounded by the problem of immune compatibity and rejection , that has led to patients being placed on long waiting lists hoping to receive an organ that can help them survive. Unfortunately, very often patients die long before they get the chance to move up on this list. Gill, J. et *al.* 2008 ploted the number of patients on waiting lists versus the donors and transplantations that have occurred from 1996 until 2007. They found that in the past 11 years, the number of people on waiting lists in Canada has nearly doubled and the number of donors has also doubled but it is still insufficient for the number of organs required [1].

Regenerative Medicine is a fast growing area that holds the promise of regenerating damaged tissues and organs through reparative techniques that stimulate previously irreparable organs into healing themselves. It is expected to lead to the creation of fully biological or biohybrid tissues and organs that can replace or regenerate tissues and organs damaged by disease, injury, or congenital malformations. Stem-cell based therapies, which are a large component of regenerative medicine, have already been harnessed for their potential to repair damaged organs. Unfortunately, although promising results have been obtained, especially in therapies that utilize bone marrow mononuclear cells also known as mesenchymal stromal cells (MSCs) or mesenchymal stem cells (MSCs), functional recovery has only be marginal. Truly effective clinical interventions will require innovative new strategies, involving modification of cells and/or cell surfaces with bio-interactive engineered scaffolds that will provide support and direction for precursor or stem cells to differentiate and affect regeneration.

Tissue engineering can be considered a subset of regenerative medicine that seeks to develop biomaterials and scaffolds to engineer functional replacement tissues or organs; broadly through three different methods as depicted in Figure 1. The first method requires the implantation of a scaffold or biomaterial, onto which cells are required to adhere within the organism to regenerate a tissue. Another method consists of injecting specific cells to a certain part of the body that are capable of regenerating the damaged tissue in that area.

Finally the last method consists of implanting a scaffold containing cells that are eventually capable of producing their own extracellular matrix to reform the tissue of interest, while the scaffold degrades and disappears [2].

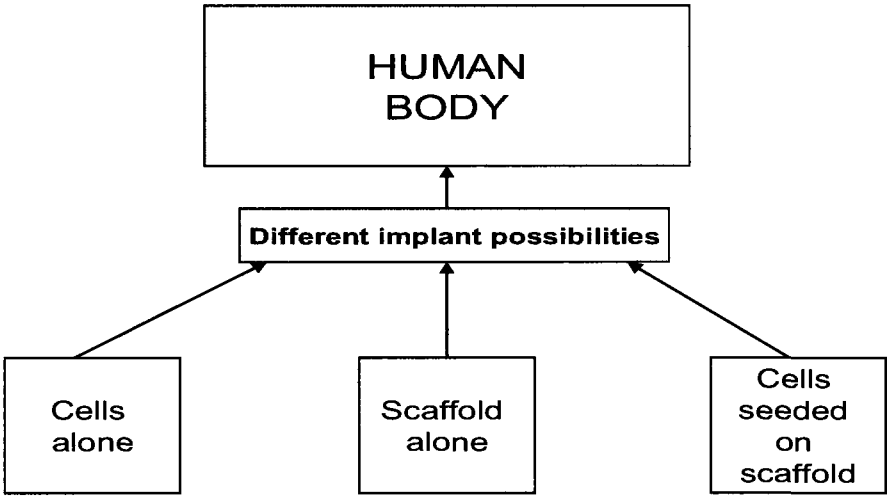

Figure 1: The different strategies of tissue engineering. Adapted from Kuo C.K. et *al.* (2003) [2].

Tissue engineering attempts to recreate natural organs or tissues. Many groups are working to regenerate skin [3], cornea [4], heart tissue [5], bone [6], regeneration of damaged spinal cord [7], cartilage tissue [8] and periodontal tissue [9].

Nowadays, the regeneration of bone tissue is of great interest to trauma and orthopaedic surgeons as limbs can be salvaged instead of amputated as were in the previous century. This kind of therapy requires the recreation of bone by tissue engineering techniques to compensate for extensively lost bone during a disease or some kind of injury [10].

The objective of this project has been to combine Mesenchymal Stromal Cells (MSCs) with type I Collagen scaffolds modified with particular peptides containing cell adhesive sequences, the CRC peptides, to regenerate bone tissue implants. The next section will explain further the different elements used in this project.

Chapter 2: Background

This section provides a detailed review on Mesenchymal Stromal Cells (MSC), type I collagen scaffolds and CRC peptides as a preface to describing the tissue engineering methodologies used.

2.1 Mesenchymal Stromal Cells

Mesenchymal stem cells are (formerly known as mesenchymal stem cells) are an important group of cells used often in regenerative medicine.

2.1.1 Characteristics of MSCs

Mesenchymal stromal cells are non-hematopoietic, stromal cells with multipotential capacities that have been regarded as adult source human stem cells. While these cells are most commonly isolated from bone marrow, they have also been isolated from other tissues. These include adipose tissue, skeletal muscle, cord blood, fetal liver, amniotic fluid, circulatory system, peripheral blood, lung, periostum, synovial membrane, dermis, and pericytes and dental pulp [11]. However the main source of MSCs commonly used in tissue engineering is bone marrow.

MSCs from bone marrow can be easily proliferated in vitro. The benefit of the multiplication capacity of these cells is that they can be extracted from the patient, allowed to divide extensively and later returned to the patient's body. Most adult stem cells are incapable of such replication capacities. And what is more is that these cells have great differentiation capacities as they can differentiate into several lineages such as adipocytes, chondrocytes, osteoblasts, myocytes, tendons, dermatocytes, marrow stroma and beta-

pancreatic islets [12,13,14,15]. Figure 2 illustrates the diverse lineages these cells differentiate into.

Figure 2: The diverse lineages that human Mesenchymal Stem Cells can differentiate into. Adapted from Kadereit, S. (2005) [16].

2.1.2 Applications of MSCs

Since MSCs have diverse differentiation capacities they have been employed in many studies having different applications. For example in an attempt to repair Spinal Cord Injury (SCI), a therapy consisting of bridging SCI regions with the use of MSCs presented by 2-hydroxymethacrylate carriers, resulted in positive effects on the Spinal Cords of these patients [7] that implied faster morphological and functional recoveries of the tissues from the injuries . Alternatively a different study found that MSCs can be used in the repair and protection of cardiac tissue from myocardial infarction [17].

After damage, cartilage self-repair does not occur effectively in nature. Furthermore cartilage damage causes severe pain and disability in affected patients, mainly older individuals and limits mobility. One attempt to regenerate cartilage was through the use of MSC seeding on silk scaffolds. Successful results were obtained as the MSC-scaffold combination resulted in neo-tissue that was very similar to the natural cartilage of the body [8].

The present study focuses on the osteogenic differentiation capabilities of MSC differentiation into osteoblasts. Osteogenesis of MSCs takes place in three steps. They first

start by adhering to a substrate and proliferating. Proliferation then stops to allow extracellular matrix (ECM) production, which envelopes the cells. Finally, the ECM becomes mineralized as the MSCs differentiate into osteocyte-like cells [8].

Several technologies have been developed to induce MSC-to-osteoblastic differentiation. For example one, a cell binding sequence known as P15 found in type I collagen was shown to promote successful differentiation of MSCs into osteoblasts [18]. Other researchers used two different rat MSC carriers, Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) and calcium-phosphate loaded scaffolds, to evaluate their capacity at promoting osteogenesis. Despite these two carriers have different surface chemistries, they were both found to promote MSC differentiation into osteoblasts [6].

The great interest in using MSCs as a source for osteoblast generation is due to the potential value of MSCs in terms of its large proliferation capacity compared to other adult stem cells, and also its ability for osteogenic differentiation as mentioned before. Human bone marrow MSCs were found in numerous studies to be successful at proliferation and differentiation into osteoblasts [19-23].

In a study comparing MSCs grown on plastic to their culture on type I collagen enriched with glycosaminoglycan scaffolds, it was found that MSC differentiation into osteoblasts was more efficient on the collagen scaffold than on plastic. Also pore size was easy to control and according to the size of the pore different osteogenic conductive patterns could be established [24]. In general, large pore size was found to better benefit osteogenic conductivity. This could be very useful as different molecules could be added to the collagen scaffold to promote better cell survival and differentiation to generate effective bone tissues.

2.2 .Collagen scaffolds

2.2.1 Collagen molecules

Collagen is the most abundant protein in mammals. It represents up to 25 to 35 % of all proteins in the body and forms the scaffolding for most connective tissues of the body.

Collagen fibrils comprise three left handed polypeptide helices that twist together to form one big tropocollagen helix that is right handed. One tropocollagen or collagen molecule measures up to 300nm and its diameter is 1.5 nm wide. Collagen molecules are composed of repeating units of Gly-A-Pro or Gly-B-Hip where A and B can be any amino acids. The abundance of glycine residues allows tropocollagen formation, as these small amino acids are usually the ones occupying the middle section of the helix since glycines are the smallest amino acids and can fit easily into this middle block [25, 26, and 27].

Despite the characterization of 28 types of collagen molecules found by researchers, only four types constitute most of the collagen found in the body. These are known as type I, II, III and IV. Each type corresponds mainly to certain regions of the body. However type I, which is also the one of interest for this project, is the overall most abundant type in humans. It is present in skin, tendon, vascular, ligature, organs and bone tissue. It can also be found in healing wounds as part of scar tissue [28, 29].

Collagen type I is of great interest in tissue engineering because of its very many advantages. According to previous research, the benefits are many and include: biocompatibility, osteocompatibility, minimal potential for antigenicity after removal of telopeptides, adhesiveness, and fibrous, cohesive and nonfriable, suturable, high porosity beneficial for neohistogenesis. Collagen fibers can be combined with other biomaterials and further incorporated into organs [30]. Their flexibility favors easy pressure relieve in tissues. The disadvantages of using this material are fewer: a potential for antigenicity through telopeptides and no inherent rigidity [30].

Other researchers have also found other benefits in using type I collagen for tissue recreation. For instance this kind of collagen plays a major role in the formation of bone from progenitor cells. Herein lies the reason for its use in this bone regeneration project. Also it plays a major role in the structure and strength of a tissue. Collagen structure also favors locomotion, adhesion, proliferation and differentiation of cells [18, 31, 32, and 33]. Type I collagen contains peptide sequences beneficial for osteogenesis and further osteoblast survival [6, 18]. A different study compared collagen scaffolds to vitronectin ones. Both matrices favored osteogenesis of MSC but in distinct manners. Whereas vitronectin favored focal adhesion formation and upregulated certain biochemical pathways, collagen reduced focal adhesion points and upregulated other pathways that eventually also led to osteoblast

formation [34]. Another paper showed that collagen scaffolds were capable of retaining osteogenic differentiation potential from early passages of in vitro growth of MSC even when these were further proliferated ex vivo [35]. Other researchers were capable of regenerating bone in damaged areas by implanting an in vitro developed transplant formed of type I collagen scaffolds seeded with osteoblasts and MSCs. Collagen was capable of both maintaining osteoblastic phenotype and differentiating MSCs into bone forming cells. Controls containing only scaffolds were unable to restore bone in damaged areas [36].

This section looked at collagen helices and their application in tissue engineering. The next section will describe how to make collagen scaffolds.

2.2.2 Type I collagen scaffolds synthesis

All these major advantages of type I collagen, exploited in previous tissue engineering studies, where collagen scaffolds were developed with the purpose of recreating, or restoring damaged tissue, have made it an ideal candidate for this bone tissue engineering project.

Therefore type I rat collagen fiber solutions were crosslinked using 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) a water soluble crosslinker depicted in Figure 3 since it was previously seen that uncrosslinked collagen matrices were unstable and degraded more easily than the ones that were crosslinked [37, 39]. Also EDC had been previously chosen in our group because it is a member of the water soluble carbodiimide (WSC) family of zero crosslinkers [37]. WSC typically function by activating carboxylic acid groups to form either ester or amide groups. The removal of EDC prevents toxicity problems such as former linkers were found to do like Glutaraldehyde or Rose Bengal [38, 39].

Furthermore the efficiency of crosslinking collagen fibers with EDC was improved substantiatly by using the N-hydrosuccinimide (NHS) activator shown in Figure 4. NHS was able to increase the crosslinking rate of EDC molecules [41].

In order to better understand the way EDC and NHS work together to form collagen networks Figure 5 illustrates their mechanism.

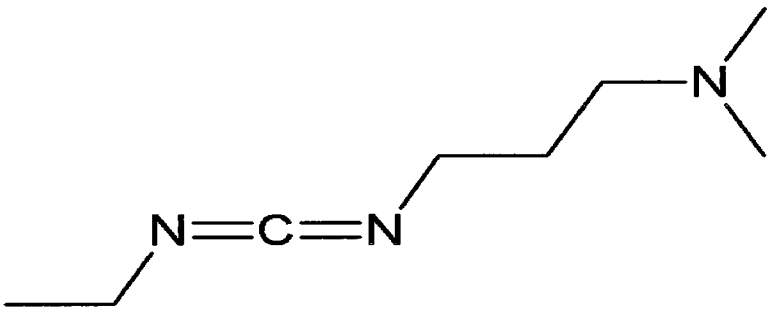

Figure 3: Chemical structure of the 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) crosslinker [40].

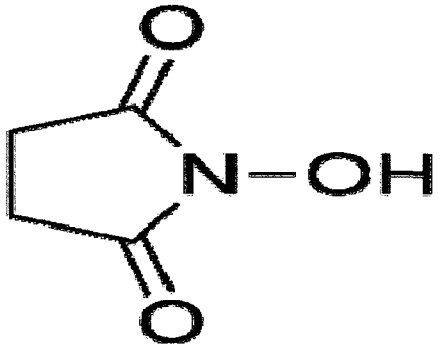

Figure 4: Chemical structure of N-hydrosuccinimide (NHS) [41].

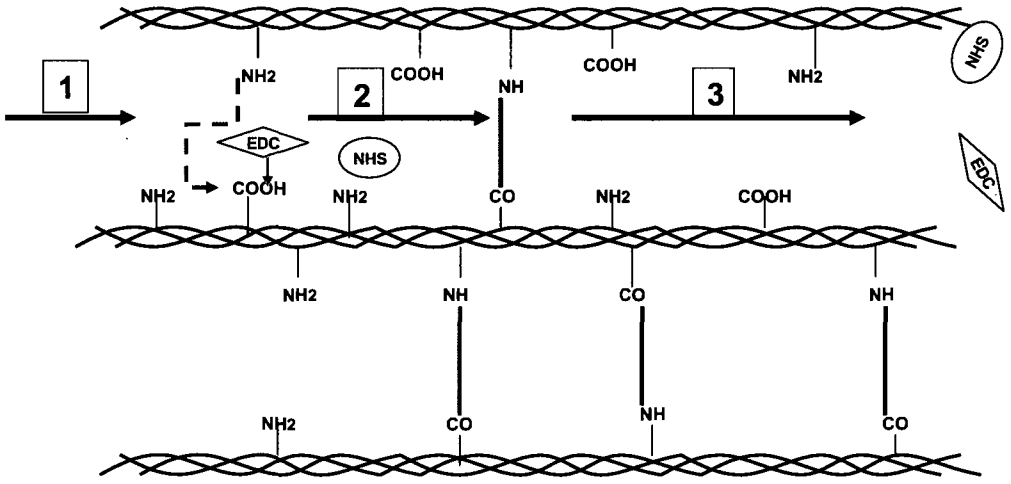

Figure 5: Crosslinking of collagen fibers by EDC crosslinker and its NHS activator. The different steps of the crosslinking are highlighted in the schematic by the numbers 1, 2 and 3. Step 1 consists of the formation of an instable o-acylisourea intermediate that is further stabilized by NHS. In step 2, a reactive amine group (on collagen surface) attacks the unstable intermediate to form and amide bond and hence releasing the EDC and NHS molecules to the surroundings (step 3).

The different steps of the crosslinking are highlighted in the schematic by the numbers 1, 2 and 3. Step 1 consists of the formation of an instable o-acylisourea intermediate that is further stabilized by NHS. In step 2, a reactive amine group (on collagen surface) attacks the unstable intermediate to form and amide bond and hence releasing the EDC and NHS molecules to the surroundings (step 3).

The collagen crosslinking mechanism illustrated above is initiated when the carbon located between the two nitrogen atoms in EDC molecules reacts with carboxylic acid groups located on glutamic or aspartic acid residues on the collagen fibers. This results in an amine-reactive intermediate denoted by o-acylisourea which is unstable in an aqueous solution. Therefore the use of NHS will help stabilize this intermediate by creating an N-acylurea group which consists of a bond between the nitrogen atom of NHS and the carboxylic acid group of a glutamic or aspartic acid residue on collagen fibers. The resulting

intermediate becomes reactive to amine groups such as lysine or hydroxylysine located elsewhere on the collagen fibers. Nucleophilic amine groups will then attack the activated carboxyl intermediate to form an amide bond. EDC and NHS are further released to the surroundings where they are precipitated out of the system by washing steps requiring several buffers. Before washing newly made scaffolds are allowed to properly crosslink at room temperature and at 37°C in the incubator for a total of 48 hours [42].

To better mimic the extracellular matrix, these collagen scaffolds were improved by addition of molecules that could better promote cell adhesion, proliferation and differentiation.

2.3 Improving collagen scaffold functionality

Improving collagen scaffold functionality is achieved by rendering the scaffolds as similar as possible to the extracellular matrix which is what facilitates cell communication. This part will review how this improvement can be achieved.

2.3.1 Importance of cell communication

Cell-substrate communication is essential for cell adhesion, proliferation, migration, and often differentiation. It is by means of molecular interactions on the surfaces of cells and in the extracellular matrix (ECM) that cell interaction with its microenvironment takes place. The types of interactions occurring are either receptor-bound element interaction or cell surface receptor with a secreted molecule that travels all the way to this receptor.

The main components of the communication system between cells and the ECM are the integrin receptors which are a large family of cell surface receptors that interact with other elements of the microenvironment such as fibronectin and laminin molecules to provoke a certain cell response [43]. What is more is that cell communication is mediated by the intermediary of specific peptide sequences located on fibronectin, laminin and vitronectin and by other molecules like signaling growth factors such as heparin that facilitate cell

interaction with other cells and with its environment, the ECM [43,44]. For example many studies have found that the RGD (Arginine-Glycine-Aspartic Acid) or RGDS (Arg-Glycine-Aspartic Acid-Serine), YIGSR (Tyrosine-Isoleucine-Glutamine-Serine-Arginine), LQVQ (Leucine-Glutamine-Valine-Glutamine), IKVAV (Isoleucine-Lysine-Valine-Alanine-Valine) peptides that can be found on fibronectin, laminin and vitronectin are capable of promoting cell adhesion by binding to the integrin receptors of the cell surfaces [43]. Hence it is this complex system of receptors and receptor binding elements that endorse the different cell activities such as adhesion, expansion, proliferation and differentiation [43].

The next section will further discuss two bioactive sequences involved in cell communication and that will be used in this project.

2.3.2 LQVQ and RGDS bioactive sequences

Although many ligands have been used to modify biomaterials, in order to improve their biocompatibility and tissue formation capacity, my project focused only on LQVQ and RGDS.

LQVQ sequences belong to the laminin family of extracellular matrix members. They were previously found to contribute to cell attachment and neuronal outgrowth as well as matrix metalloproteinase secretion that promoted nerve repair [45]. A different paper classified the laminin-1 bioactive sequence, LQVQ as an active sequence having an important biological role and is a key element with the laminin cell receptors [47].

RGD or RGDS peptides from fibronectin and other ECM molecules have been extensively studied for their cell-adhesion promoting activity. RGD sequences enhance cell binding by activating certain integrin receptors [47]. Silicone surfaces modified with RGD peptides had better cell binding than tissue culture plates and non modified silicone surfaces [48]. Furthermore the way the RGD or RGDS sequence is presented to the cells on the biomaterial can elicit different responses by these latter ones. For example 440 nm spacing is enough for integrin mediated fibroblast spreading, while a distance of 140 nm can illicit the production of focal contact points and stress fibers [49].

Flanking sequences surrounding the RGD peptide also have an effect on cell response as they can alter the binding affinities to cell surfaces [50, 51].

Also another study showed that fibronectin is capable of taking different conformations when coated on different surfaces such as in the case of polystyrene. [52]. For this reason potential ligands containing the bioactive sequences of interest can have a different activity than predicted once bound to the biomaterial in use as their conformation can be changed.

Additionally the density of the bioactive peptide RGDS (as well as other peptides) on tissue engineered scaffolds were found to affect the cell adhesion, proliferation and differentiation capacities. Smooth muscle cells adhered better and proliferated less on surface modified scaffolds with peptides, compared to the non-modified controls. It was also found that they produced less matrix on ligand modified surfaces compared to the controls [53]. Another paper also found that the average RGD density dictated migration of NR6 fibroblastic cells. This group also found that clustering of the RGD peptide enhanced cell migration speed [54]. Alternatively another group compared the use of long peptides containing repeating RGD units to shorter ones containing single RGD units. They found that the peptide conformation could dictate cell spreading and differentiation abilities. Hence adjusting the peptide size and the number of bioactive sequences it contains has the ability to alter surface RGD density [51].

The ligand type, density and how it is presented are all parameters that were found to influence cell adhesion, expansion and possibly differentiation. Although collagen contains RGDS sequences, the effect of enhancing the RGDS content of collagen-based scaffolds to increase ligand density was examined, using MSCs as test cells. The interaction between LQVQ and RGDS was also examined.

2.3.3 CRC peptides

2.3.3.1 Structure of CRCs

Members of Dr. Jim Harden's lab at the University of Ottawa, designed a de novo series of self assembling peptides denoted CRC (Coil Random Coil) that contained several types of bioactive sequences [55]. These peptides have the advantage of being able to introduce guided modularity to the system; to produce designer/tailor-made microenvironments i.e., simply changing the peptides can change the "micro-environment" to suit target cells. For example the densities on collagen scaffolds of the RGDS and LQVQ sequences can be increased and the interaction of the two bioactive sequences on cells can be studied, when these bioactive sequences are included in the CRC peptides, further used to modify the collagen scaffolds. As a matter of fact, CRCs were designed with hydrogel self assembling capacities to add a monolayer or more of bioactive sequences to any biomaterial surface, increasing by this way the concentration of the ligands.

These peptides were first tested on glass substrates for their efficacy at forming a monolayer on this substrate and their biocompatibility capacity. By increasing the RGDS and LQVQ densities on this kind of material, cell adhesion and proliferation were in turn improved accordingly to the ligand densities and to the type of cells cultured [55]. However the competitive adsorption that arose from serum proteins kept destabilizing the CRC hydrogel, causing its removal from the surface. Therefore the weakness of the hydrogel's stability on the glass substrate surface led to the interest in covalently crosslinking CRC peptides to collagen scaffolds and testing its effect [55].

A better understanding of CRC peptides requires a description of their structure and their hydrogel formation abilities. First these peptides are formed by two identical blocks denoted as Coil (thus the C in CRC) which is ampholytic leucine zippers characterized by a repetitive heptad pattern. This repetitive heptad pattern has hydrophobic amino acids, mainly leucine, that alternate with charged residues mainly glutamine and lysine. These identical blocks contain associating ends that preferentially allow formation of heterotrimer bundles that assemble into hydrogel networks. The association between two ends consists of a hydrophobic interaction between the end of one peptide and that of another. CRCs were designed so that the ends of the same peptide do not associate with each other as they have the same orientation which makes them entropically unfavourable if they were to come together [55]. Hydrogel formations occurred at moderate temperatures and pH conditions

that favoured leucine zipper domains to associate into amphiphilic alpha helices that further promote the self assembly of the hydrogel.

CRCs also contain one middle block denoted as Random (thus the R in CRC). The Random section is composed of a repetition of $(Ala\text{-}Gly)_3$-Pro-Gly-Gly. Amongst these sequences of amino acids, a single bioactive sequence is included. This sequence could be any sequence however for this project RGDS and LQVQ were preferentially chosen. Figure 6 illustrates the structure of a CRC hydrogel on a random scaffold surface. The scaffold is the flat part on the bottom of image. The CRC hydrogel corresponds to the network of peptides on top of that flat layer. It can be seen that the CRC peptide contains two coil areas (that were depicted as two bold lines at each extreme of the peptide for simplicity purposes) on each extreme which correspond to the leucine zippers described above. The non coil middle block (depicted as a curve with a red or yellow colored disk) represents the Random area containing the bioactive sequence right in the middle of this part. Once in hydrogel form, the helical part (the C part) of each CRC is engaged with 2 other helical parts from 2 different peptides or the biomaterial at the base.

The secondary structure of the proteins and the mechanical properties of the formed hydrogels were not adversely affected by the presence of the RGDS (or LQVQ) sequences. Furthermore adding RGDS bioactive sequences to the middle block of CRC peptides was found to promote adhesion, spreading and polarization of fibroblast cells on surfaces modified with these peptides [55].

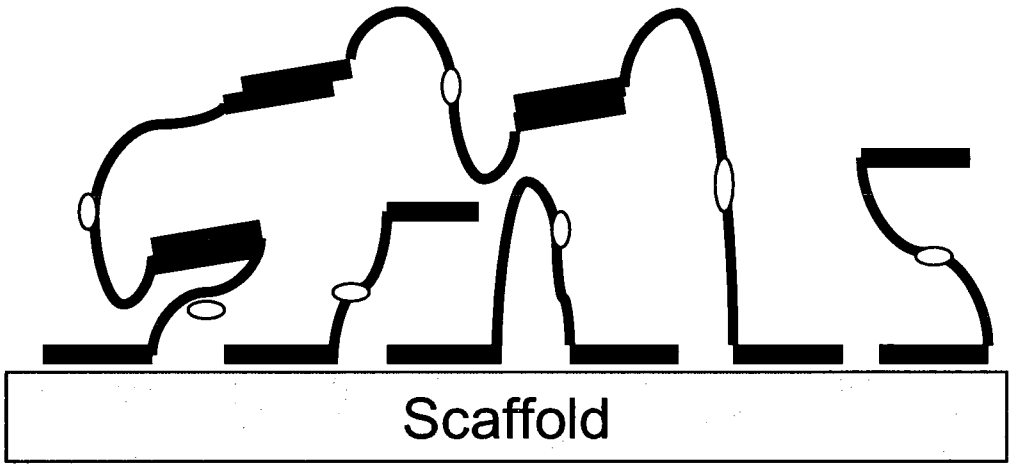

Figure 6: CRC hydrogel structure when grafted onto the surface of a scaffold (in grey at the bottom) [55]. Adapted from Fischer et *al.* (2007). One CRC peptide is composed of two helical areas formed of leucine zippers and one middle block (non coil area), the Random block that contains the bioactive sequence in the middle (depicted in either red or yellow in Figure).

2.3.3.2 Covalent binding of CRCs

In this project CRCs were covalently bound to the collagen scaffolds via either Sulfo EGS (Ethylene glycol bis [sulfosuccinimidylsuccinate]) crosslinking, EDC or EDC/NHS crosslinking. The mechanism by which EDC crosslinked CRCs to the surface of collagen scaffolds is very similar to the one described above for the crosslinking of collagen fibers. The difference is in the fact that instead of having two collagen fibers crosslinking to each other, there is one CRC peptide that is crosslinked or covalently bound to the surface of a collagen scaffold. Figure 7 illustrates this process.

CRCs were also bound to collagen by using the homobifunctional Sulfo EGS crosslinkers since they have two identical functional groups at each end of the molecule. Figure 8 illustrates the chemical structure of sulfo EGS. Binding with Sulfo EGS involves a

similar to EDC mechanism, however in this case the linker is not removed from the system. In fact sulfo EGS serves as a binding arm that brings the CRC peptide to the collagen scaffold surface.

The reaction between the sulfo EGS molecule and the collagen surface (or CRC peptide since the reaction is the same) starts with an amine group of collagen (or CRC) attacking the carbon atom from the ester group of the sulfo EGS. This results in an amide bond between the collagen (or CRCs) and the sulfo EGS. Figure 9 illustrates this mechanism.

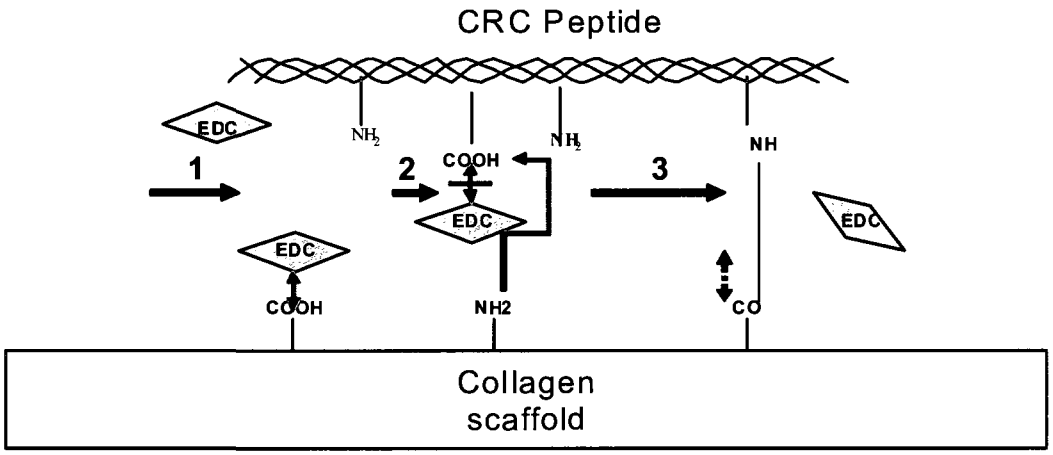

Figure 7: Covalent grafting of CRC peptides to the surface of a collagen scaffold with EDC crosslinker.The different steps are highlighted by the numbers 1, 2 and 3. Step 1 consists of the formation of an unstable o-acylisourea intermediate by the reaction of EDC with a carboxylic acid group on either collagen surface or CRC peptide. In the next step (2) a reactive amine group (on either substrate collagen or CRC) attacks the unstable intermediate to form and amide bond and hence releasing the EDC to the surroundings (step3).

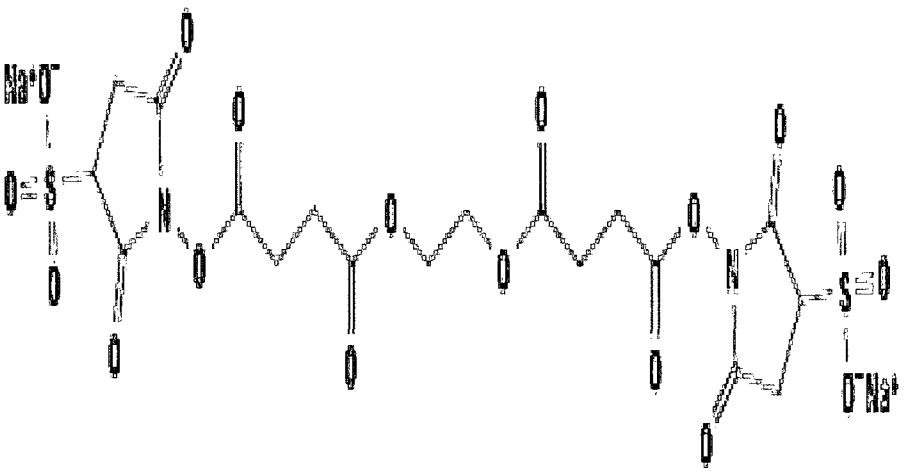

Figure 8: Chemical structure of the Sulfo EGS crosslinker [56].

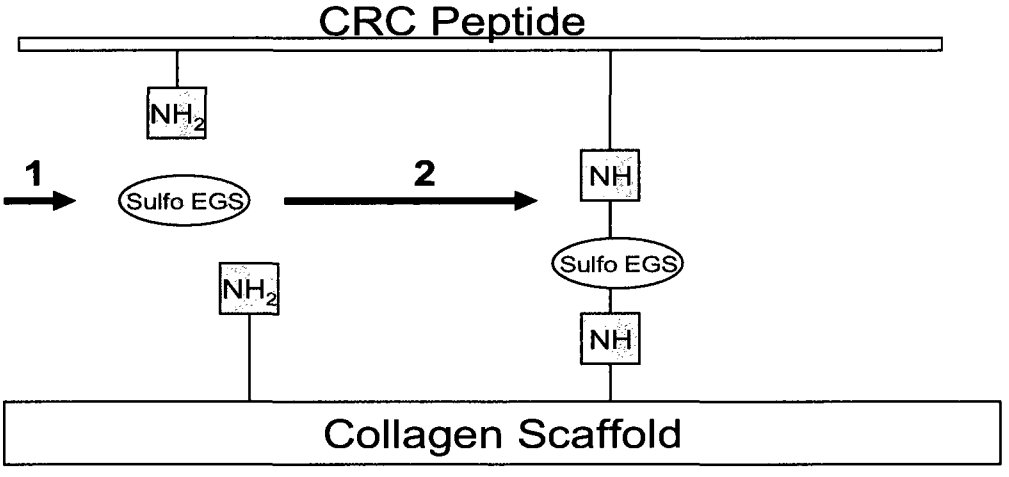

Figure 9: Covalent grafting of CRC peptides to the surface of a collagen scaffold via Sulfo EGS. The different steps are highlighted by the numbers 1 and 2. Step 1 starts when the amine group on either CRC peptides or collagen scaffolds attacks the carbon atom from the ester group of the Sulfo EGS. This results in an amide bond between the collagen (or CRCs) and the sulfo EGS. The other end of the Sulfo EGS also reacts with

another amine group (located on either collagen or CRC, step 2) to form another amide bond.

Chapter 3: Objective and Scope of Project

The objective of this project was to investigate the use of CRC peptides for modification of an existing scaffold in this case, collagen hydrogel. The biological responses of MSCs to the peptides related to their differentiation into bone were examined. Interaction between RGDS and LQVQ peptides, *eg.*, possibly synergistic or antagonistic were characterized. Briefly MSCs were cultured for 2 weeks on collagen scaffolds modified with four types of combinations of CRC peptides. The first one consisted of CRC peptides without any bioactive sequence for control purposes. (To avoid confusion these control peptides were denoted P-CRC for the rest of this report). Despite the fact that collagen fibers contain RGDS groups of their own, it is hypothesized here that scaffolds with P-CRCs should not be able to improve cell adhesion by much, as the RGDS and LQVQ functional groups are not as abundant on these controls as they should be on samples modified with CRC-RGDS and/or CRC-LQVQ. The second scaffold was modified with P-CRC and CRC-RGDS at a 1:1 (w/w) ratio to see the effect of the bioactive sequence (RGDS) which is thought to promote cell adhesion. A third sample had a similar purpose as the second sample but it contained P-CRC and CRC LQVQ (LQVQ is known to promote cell adhesion) at a 1:1 (w/w) ratio. Finally the last sample tested the combined effects of collagen scaffolds modified with CRC-RGDS and CRC-LQVQ at a 1:1 (w/w) ratio. Previously other members from our group found that the combination of RGDS and LQVQ containing peptides had the potential to promote neuronal stem cell proliferation but to retain stem cell phenotype. In this project we are also interested to see if this peptide combination has the same results on MSCs in terms of proliferation and differentiation. Being able to control proliferation and differentiation on a biomaterial is an important part of tissue engineering, as cell growth on scaffolds can be determined as needed.

Also controls lacking collagen scaffolds were prepared. One had a mixture of peptides only, CRC-RGDS and CRC-LQVQ at a 1:1 (w/w) ratio, on a polystyrene well of a 48 well plate to evaluate the effect of the peptides alone. The other control consisted of an empty well of the 48 well plate used for cell culturing to determine the efficiency of the MSCs grown on their own.

MSC proliferation was counted manually in a blinded study and cell numbers were recorded and can be found in later in this report. Osteogenesis was tested by observing the mineralization abilities of MSCs on the altered biomaterials. Calcium depositions were observed with alizarin red and osteopontin markers were used to further determine the osteoblastic phenotypes.

CRC peptides were also characterized on the collagen scaffolds to establish their grafting abilities to the biomaterial. For that ^{125}I radiolabelling of P-CRC and CRC-RGDS by their tyrosine residues was also done. First radioactive peptides were loaded on to collagen scaffolds surfaces and loading efficiencies were compared to evaluate whether the additional bioactive sequences could change the structure of the entire CRC peptide molecule. Since peptide loading onto collagen surfaces was found to be very similar, all other chemical characterization studies used only P-CRCs. Adsorption of P-CRCs was also modeled to obtain the adsorption properties for the collagen scaffolds (more on this will be discussed in the Materials and Methods section).

A different study with radiolabelled P-CRC looked at differences amongst the two crosslinking chemicals used for characterization properties of CRC peptides. EDC (or EDC with an activator NHS) and Sulfo EGS were the two crosslinkers compared for their efficiency for covalently binding the peptides to collagen. However since EDC resulted to be more efficient at grafting CRCs, it was the only one used for grafting peptides on collagen scaffolds for cell culturing. In this same experiment washing samples with a harsh buffer, 10% SDS-PBS, a pre-step required in cell culturing, was examined for its effect on the amount of peptide retained on collagen scaffolds. Another experiment also tested the efficacy of the grafted P-CRC peptides when submerged in cell culturing conditions (10% FBS) for 336 hours.

Chapter 4: Materials and Methods

4.1 Substrate preparation

4.1.1 Collagen Solutions

Type I from porcine atelocollagen was purchased from Nippon Meat Packers Inc. (Tokyo, Japan). One milligram of collagen was weighed into a round bottom flask. Double distilled water was then added to give the desired 10% (w/w) ratio of collagen. The flask was then covered with Parafilm and placed in the refrigerator overnight to allow proper dissolution of the collagen in the water. The following day a glass rod was inserted into the collagen solution and the glass rod-flask system was placed into a mechanical mixer from VWR (USA) to allow proper mixing of the solution. It was then left to mix for about a week, or until a homogeneous viscous collagen solution was obtained, at 4°C covering all open surfaces with Parafilm to prevent any source of contamination.

This viscous solution was then transferred into a sterile 10 ml syringe with no plunger. The bottom part of the syringe was covered with syringe tip septum and Para filmed to prevent leaking from the system. The 10 ml syringe was then centrifuged three times for 20 minutes each period, at 5000 rpm and at 4°C in a 20.1 JL Rotator placed into an Avanti J.25 Centrifuge from Beckman. Centrifuging helped minimize the formation of bubbles in the solution. The plunger was reinserted into the syringe and then the solution was stored at 4°C until needed.

4.1.2 Collagen Scaffolds

Manufacturing of collagen scaffolds was a process requiring several steps. The first step consisted of mixing about 0.5 ml of a 0.05M Morpholinethanesulfonic acid solution (MES) from EMD Chemicals Inc.(USA) (containing 5 mg of Alizarin Red S Monohydrate

from Sigma –Aldrich Canada Ltd (Oakville, Ontario, Canada) for every 100ml of 0.05M MES solution), with 0.5 ml of 10% collagen gel into a T-system from SciPro Inc. Note that all mixing steps were done in icy water in containers. Bubbles trapped in the T-system were removed by tapping onto system. Then a 1N NaOH solution from EMD Chemicals Inc.(USA) solution was added slowly until pH reached 5.5 i.e. when solution in T-system turned pink. Typically 27-30 ul of NaOH solution was enough to reach the desired pH. The next step consisted of adding N-hydrosuccinimide (NHS) purchased from Fluka (Buchs, Switzerland). Once NHS was added the solution in the T-system was left in the icy water for for 15 minutes in order to promote proper dispersion of the NHS molecules throughout the mixture prepared so far.The amount of NHS was calculated based on EDC:NHS ratio of 0.35, which was predetermined. Finally the crosslinker, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) purchased from Sigma –Aldrich Canada Ltd (Oakville, Ontario, Canada), was added to the mixture and mixed thoroughly and very quickly before crossklinking occurred. The amount of EDC added to the system, was based on a ratio of EDC:collagen equal to 0.7. After proper but very quick mixing, the collagen solution was immediately removed from the T-system and placed on a glass slide containing spacer elements to create a thickness of 500μm for the collagen scaffolds. Once the collagen was placed on the first glass slide, the second glass slide was used to flatten solution and create a plane scaffold sheet.

Molded gels were first left in a plastic container, in which a water beaker was placed, for 24 hours. Then the entire plastic container was placed in an incubator for another 24 hours. After this, gels were demolded and further underwent a washing treatment.

Collagen scaffolds were then immersed in 0.1M Na_2HPO_4 solution for 1 hour at 4°C to facilitate the removal of any excess EDC. Gels were further washed with 10mM Phospate Buffered Saline solution (PBS) for one week, changing the buffer solution 2 to 3 times a day. Finally gels were stored in glass vials containing 10mM PBS. One per cent of chloroform was added to the PBS to maintain sterility.

4.2. Surface modification of collagen scaffolds for chemical characterization

4.2.1 CRC peptides

4.2.1.1 Sources of CRC peptides

CRC peptides were provided by Stephen Fisher from the Harden group at the University of Ottawa.

4.2.1.2 Types of CRCs

There were 3 different types of CRCs used in this project. Complete peptide sequences can be found in Appendix A. The first type of peptide was the control CRC that lacked any bioactive sequence denoted P-CRC. The next one was the CRC-RGDS peptide containing one fibronectin bioactive sequence called RGDS in the R middle block. Finally the last CRC molecule used in this study had one laminin LQVQ bioactive sequence in its R area and hence denoted as CRC-LQVQ. The molecular weight of each of these peptides was 17902 g/mol, 18318 g/mol, 18840 g/mol, for P-CRC, CRC-RGDS and CRC-LQVQ respectively.

4.2.2 Radiolabelling of CRC peptides with ^{125}I

CRC-RGDS and P-CRC were labeled with ^{125}I vthrough their tyrosine residues in two different experiments: one for CRC-RGDS and another for P-CRC. Since it is the same procedure for both peptides, only CRC-RGDS will be mentioned from now on for simplicity. ^{125}I was obtained in the form of $Na^{125}I$ from MP Biomedicals (Irvine, CA, USA). Radiolabelling of CRC-RGDS with ^{125}I was done by the IODO-GEN Pre-coated Iodination Tubes from Pierce (Rockford, Il, USA) according to the Chizzonite indirect method recommended by the manufacturer [57]. Briefly, 0.25 mCi of $N^{125}I$ was mixed with 100ul of tris iodination buffer (TIB, 25mM Tris-Cl, 0.4 M NaCl, and pH 7.5) in an IODO-GEN tube and allowed to activate for 6 minutes at room temperature. Subsequently, the activated

iodine was removed and mixed with 100 μL of a 10 μM CRC-RGDS solution in TIB and reacted for 8 minutes. The reaction mixture was then loaded into pre-equilibrated D-Salt Polyacrylamide Desalting Column from Pierce (Rockford, Il, USA) to separate radiolabeled CRC-RGDS from free ^{125}I. Radiolabeled peptide was eluted from the column by six 1 mL additions of of a TIB solution also containing 5 mM EDTA and 0.05% sodium azide (EDTA and sodium azide are added as preservatives). Eluate was collected into 1.5 mL screw cap tubes and stored at 4°C until further use. A specific activity for the labeled peptide of approximately 1 x 10^8 cpm/mL was obtained.

4.2.3 Loading of radiolabeled CRC peptides on collagen scaffolds surfaces

4.2.3.1 For characterization of differences amongst CRC peptides with and lacking bioactive sequence

This first surface characterization test was used to quantify how much peptide was loaded into the collagen scaffolds and to determine whether the embedded RGDS bioactive domain affected this loading. In preparation for the test, stock solutions of P-CRC and CRC-RGDS of known peptide concentration and specific activity were prepared. To prepare these stock solutions, unlabeled P-CRC and CRC-RGDS were first dissolved in 10 mM phosphate buffer (pH 7.5) at a peptide concentration slightly higher than 100 μM. Then, the P-CRC and CRC-RGDS solutions were spiked with ^{125}I labeled P-CRC and CRC-RGDS, respectively, such that a final peptide concentration of 100 μM and a final activity of approximately 2 x 10^6 cpm/mL were obtained. Subsequently, the radioactive stock solutions of P-CRC and CRC-RGDS were each serially diluted with 10 mM phosphate buffer (pH 7.5) to obtain solutions of the following peptide concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 μM. BSA-coated tubes were used to prevent loss of the peptide during serial dilutions. To perform the peptide loading test, triplicate collagen scaffolds cut into small disks were completely immersed in these peptide solutions overnight at 4°C. The next day, scaffolds were removed from the peptide solutions and dipped briefly into water to remove

the excess peptide. After blotting the scaffold samples dry, the radioactivity of each scaffold was counted for 2 mins in a Packard Cobra Quantum gamma counter (Perkin Elmer Inc.). The mass of peptide detected on the scaffolds was calculated by comparing the radioactivity of the samples with that of the initial 100 μM peptide stock solutions.

The fitting of the data was tested by two adsorption models, i.e. the Freundlich and Langmuir model. Since the fit was better by the Freundlich model (Equation 1), only this model was used.

$$q = k_F \times C^n \qquad \textbf{Equation 1}$$

Where q is the amount of adsorbed peptide in mol/g, C is the peptide concentration in solution mg/L, k_F is the adsorption constant that indicates the capacity of the substrate for adsorbing the peptide. It has $mol^{1-1/n}g^{-1}L^{1/n}$ as its unit. n is a dimensionless coefficient indicating the affinity of the peptide to the substrate. k_F and n represent the Freundlich coefficients. [58].To plot the data and obtain the required parameters, it is necessary to linerarize Equation 1.

$$\text{Ln } q = \ln k_F + n \ln C \qquad \textbf{Equation 2}$$

The Freundlich coefficients are then obtained from the plot of ln C in the x-axis versus Ln q in the y-axis. These values are analyzed in the discussion.

Since P-CRC and CRC-RGDS had similar properties, from here on only P-CRC will be used for the rest of the chemical characterization of collagen scaffolds.

4.2.3.2 For stability study in washing solution following crosslinking of P-CRC to 10% collagen films

The second surface characterization test was used to compare crosslinking methods for stabilizing the CRC peptides loaded into collagen scaffolds. Different crosslinking methods described in the following were compared under stringent washing conditions.

4.2.3.2.1 Via EDC & EDC/NHS

A 50 μM solution of P-CRC containing approximately 2 x 10^6 cpm/mL of radioactivity was prepared in 10 mM phosphate buffer at a pH of 7.5. Triplicate collagen scaffolds cut into small disks were then loaded with P-CRC by completely immersing the disks in peptide solution overnight at 4°C. The next day, the scaffolds were removed from the peptide solution and dipped briefly into water to remove excess peptide. To crosslink the P-CRC, peptide-loaded scaffolds were then completely immersed into an EDC alone solution or an EDC combined with NHS solution overnight at room temperature. The EDC and NHS solutions were prepared in water at concentrations of 200 and 1600 uM (for EDC) and 50 and 400uM (for NHS). The scaffold samples were then removed from cross-linker (EDC or EDC/NHS) solution and dipped in 1X PBS to remove excess cross-linker (and/or activator). To test the stability of EDC and EDC/NHS crosslinked peptides under stringent conditions, the scaffolds were further incubated in 10% SDS-PBS for 2 days at room temperature. During this incubation period, the buffer solution was changed four times. Upon completion of the experiment, the amount of peptide remaining in the scaffolds was determined by counting the radioactivity of each scaffold sample for 2 minutes.

4.2.3.2.2 Via Sulfo-EGS

To pre-activate collagen scaffolds for crosslinking to P-CRC, triplicate scaffolds cut into small disks were completely immersed in a solution of 1.5 mM Sulfo-EGS for a period of 2 hr on ice. To prepare the crosslinker solution, Sulfo-EGS (Pierce) was dissolved in either double distilled water or 1X PBS. After rinsing them 3 times for 5 minutes each time with either water or 1X PBS, the Sulfo-EGS-modified scaffolds were then completely immersed in a radiolabeled P-CRC peptide solution overnight at 4°C. The P-CRC solution was prepared at 50 μM in 10 mM phosphate buffer (pH 7.5) and contained approximately 2 x 10^6 cpm/mL of radioactivity. The following day, the samples were removed from the

peptide solution and dipped briefly into PBS to remove excess peptides. To test the stability of Sulfo-EGS crosslinked peptides under stringent conditions, the scaffolds were further incubated in 10% SDS-PBS for 2 days at room temperature. During this incubation period, the buffer solution was changed four times. Upon completion of the experiment, the amount of peptide remaining in the scaffolds was determined by counting the radioactivity of each scaffold sample for 2 minutes.

4.2.3.3 For characterization of stability in serum following crosslinking of P-CRC to 10% collagen films

The third surface characterization test was used to assess the stability of CRC peptides crosslinked to the collagen scaffolds using either EDC or EDC/NHS under cell culture conditions. For this purpose, a 50 μM solution of P-CRC containing approximately 1 x 10^6 cpm/mL of radioactivity was prepared in 10 mM phosphate buffer at a pH of 7.5. Triplicate collagen scaffolds cut into small disks were then loaded with P-CRC by completely immersing the disks in peptide solution overnight at 4°C. The next day, the scaffolds were removed from the peptide solution and dipped briefly into water to remove excess peptide. To crosslink the P-CRC, peptide-loaded scaffolds were then completely immersed into an EDC alone solution or an EDC combined with NHS solution overnight at room temperature. EDC and NHS were prepared in double distilled water and concentrations of these two chemicals were varied at 100, 400 and 1600 uM (for EDC) and 25, 100 and 400uM (for NHS). The next day, samples were removed from crosslinker solutions and dipped in 1X PBS to remove excess crosslinkers. The samples were then incubated in cell culture media at 37°C. Cell culture media consisted of RPMI 1640 (Invitrogen) with pen/strep and 10% FBS. Sodium azide was also added to the media to a final concentration of 0.5 mg/mL to act as a preservative. At the desired times, collagen scaffolds were removed from the cell culture media and dipped briefly into 1X PBS. After blotting the scaffold samples dry, the radioactivity of each scaffold was counted for 2 mins.

4.2.4 Radioactivity emission measurement of collagen scaffolds

Radioactive emission was measured with a Packard Cobra Quantum gamma counter from Perkin Elmer.

4.3 Surface modification of collagen scaffolds for cell culturing

Collagen scaffolds were also grafted with CRC peptides for cell culturing experiments. The following are the different 7 samples prepared of modified collagen and controls: P-CRC, P-CRC/CRC-RGDS, P-CRC/CRC-LQVQ, CRC-RGDS/CRC-LQVQ, EDC modified only collagen scaffold, control consisting of only CRC-RGDS and CRC-LQVQ peptides deposited in a well of a 48 well tissue culture plate at a 1:1 ratio and a final control consisting of an empty well in the 48 well tissue culture plate.

4.3.1 Grafting of CRC peptides on collagen scaffolds surfaces via EDC

Collagen hydrogels were precut into discs to fit the wells of a 48 well tissue culture plate. Once collagen discs were loaded on the wells of the polystyrene plate, a small amount of a 1mg/ml CRC peptide solutions (the different peptide solutions made to modify collagen scaffolds and the control sample outlined above were prepared in 1X PBS and so that each peptide in every solution prepared had a final concentration of 1mg/ml in the final CRC solutions) were poured onto scaffolds until they were fully immerged in solution and left overnight at 4°C. The next day, peptide solutions were pipetted out of the wells and discarded. Scaffolds and peptide only well controls were then washed at room temperature, three times for five minutes each time, with 1X PBS solutions to remove excess peptide solutions. Enough EDC was then added to the scaffolds and to the control peptide only well

until it covered the samples completely and left for 4 hours at 4°C. The EDC solution had a concentration of 0.105 mg/ml based on a 10:1 ratio required of EDC to CRCs. After soaking samples in the EDC solution, this one was then pipetted out of the wells containing the collagen scaffolds and peptide only control well. These later ones were washed once with 1N Na_2HPO_4 (enough of this solution was added to cover completely the scaffolds and peptide only control well) for 20 minutes then 3 times with filtered 1X PBS for 5 minutes each time. To prevent any kind of contamination of samples while storage, samples were stored at 4°C in 1X PBS Chloroform (at 1% chloroform) solution. This solution was directly added to all wells containing collagen scaffolds and to the two controls containing peptides only and empty well control. Samples were then stored for further use.

4.4 Biological Surface Characterizations

4.4.1 Cell culture studies

4.4.1.1 Mesenchymal Stromal Cells: source of MSCs.

MSCs used in this project were provided from the Tulane Center for Gene Therapy. These cells were originated from human bone marrow sources.

4.4.1.2 MSC proliferation on collagen scaffolds

4.4.1.2.1 Culture medium for proliferation

MSCs were plated at an initial density of 1000 cells per well. Cells were counted with a hemocytometer from Invitrogen (Canada). MSCs were grown in a special culture medium containing alpha-MEM with L-glutamine from Invitrogen GIBCO, heat inactivated FBS at a concentration of 16.5% from Sigma Aldrich (USA), another 2mM L-glutamine solution made in 0.85% NaCl (known as Gluta-MAX-1) from Invitrogen/GIBCO, penicillin at a

concentration of 100 units/ml and streptomycin at a concentration of 100 ug/ml both from from Invitrogen GIBCO. Media was changed every 2 days and samples were observed with a phase contrast microscope from Nikon (Nikon Eclipse TE2000-E, Nikon Canada Inc.)and pictures were taken every other day.

The viability of cells was assessed using the LIVE/DEAD Viability/Cytotoxicity Kit from Invitrogen. At days 7 and 14, live/dead staining was performed in one well for each sample. Calcein AM is hydrolyzed by esterases present in live cells, yielding a fluorescent green product. Ethidium homodimer-1, on the other hand, fluoresces red in dead cells under ultraviolet light; this red stain is only able to enter dead cells since the integrity of their plasma membranes is compromised. Nuclei of the cells were stained by Hoeschst 33342 also from Invitrogen that emitted a blue color when labelled to the DNA. The stain was prepared in the dark and consisted of PBS, Calcein AM, ethidium homodimer-1, and Hoechst 33342 mixed at a ratio of 99.65 : 0.15 : 0.1 : 0.1 v/v for a final volume of 1mL . The cells were incubated in the staining solution in the dark for 20 minutes at room temperature. They were then washed twice for 10 minutes each time with PBS. Pictures were taken of the live/dead staining and then all samples were fixed in 4% paraformaldehyde (PFA) for 5 minutes.

4.4.1.2.2 Immunohistochemistry

For immunofluorescent staining with CD117 (mouse, anti-human from Chemicon International) for the MSCs, a standard protocol was used and it consisted of washing the samples 3 times for 5 minutes each time with Tris Buffered Saline (TBS) solution at a pH 8.0.

A 50 mM solution of ammonium chloride was added next, and samples were incubated for 30 minutes at room temperature to quench autofluorescence of the collagen. After quenching, a blocking solution consisting of 1% BSA, 4%FBS in TBS was added to samples and incubated for 1 hour at room temperature.

The primary antibody was diluted to 1:100 in TBS with 0.3% Triton-X and then added to the MSC samples and incubated overnight at 4°C. The next day, the primary antibody was removed and samples were washed 3 times for 5 minutes each time in TBS.

The secondary antibody (Cy3 rabbit, anti-mouse - diluted in TBA w/0.3% Triton-X as well) was then added on samples which were left at room temperature in the dark for 1 hour. Then samples were washed again 2 times for 5 minutes each time in TBS. A 1:10,000 dilution of DAPI in TBS was added to the wells, incubated for 5 minutes in the dark, then washed off 3 times for 5 minutes each time with TBS. Samples were then visualized by fluorescence microscopy.

4.4.1.3 MSC differentiation on collagen scaffolds

4.4.1.3.1 Culture medium for differentiation into osteoblasts

Cells were plated at an initial density of 1000 cells per well. MSCs intended for osteoblast differentiation were grown in medium containing DMEM, heat inactivated FBS at a concentration of 16.5%, L-ascorbic acid 2-phosphate sesquimagnesium salt, dexamethasone, beta-glycerophosphate all from from Sigma Aldrich Canada Ltd. (Oakville, Ontario, Canada), and penicillin at a concentration of 100 units/ml and streptomycin at a concentration of 100 ug/ml.

Media was changed every 2 days. Pictures were taken every other day. At days 7 and 14, live/dead staining was performed in one well for each sample. Pictures were taken of the live/dead staining and then all samples were fixed in 4% Paraformaldehyde (PFA) for 5 minutes. Samples were then washed 3 times for 5 minutes with double distilled water.

4.4.1.3.2 Immunostaining for identification of differentiation

4.4.1.3.2.1 Alizarin red

Samples were stained for osteogenic differentiation, specifically mineralization using Alizarin Red. Samples were washed twice with double distilled water, at 5 minutes each time. Alizarin Red S stain at a 2% concentration (w/v) was added and samples were incubated at room temperature for 20 minutes. Samples underwent a thorough washing

process with double distilled water several times until water became clear as opposed to pink. Samples were then observed under inverted microscope for evidence of calcium deposits.

4.4.1.3.2.2 Markers

4.4.1.3.2.2.1 CD117

For immunofluorescent staining with CD117 (mouse, anti-human from Chemicon International) for the MSCs, a standard protocol was used and it consisted of washing the samples 3 times for 5 minutes each time with Tris Buffered Saline (TBS) solution at a pH 8.0.

A 50 mM solution of ammonium chloride was added next, and samples were incubated for 30 minutes at room temperature to quench autofluorescence of the collagen. After quenching, a blocking solution consisting of 1% BSA, 4%FBS in TBS was added to samples and incubated for 1 hour at room temperature.

The primary antibody was diluted to 1:100 in TBS with 0.3% Triton-X and then added to the MSC samples and incubated overnight at 4°C. The next day, the primary antibody was removed and samples were washed 3 times for 5 minutes each time in TBS. The secondary antibody (Cy3 rabbit, anti-mouse - diluted in TBA w/0.3% Triton-X as well) was then added on samples which were left at room temperature in the dark for 1 hour. Then samples were washed again 2 times for 5 minutes each time in TBS. A 1:10,000 dilution of DAPI in TBS was added to the wells, incubated for 5 minutes in the dark, then washed off 3 times for 5 minutes each time with TBS. Samples were then visualized under a fluorescence microscope.

4.4.1.3.2.2.2 Osteopontin

The same protocol was followed for osteopontin (an osteoblast marker) detection. The only difference this time is that the osteopontin, the primary antibody, was diluted at

1:150 in TBS with 0.3% Triton-X. And the secondary antibody was Texas Red and it was diluted at 1:500 TBS with 0.3% Triton-X.

4.5 Statistical Analysis

One Way and Two Way ANOVA tests were done to evaluate statistical significance of results. Tukey/ Scheffe tests compared controls to all samples individually and determined equal significance of samples [59].

Chapter 5: Results

5.1 Chemical Surface Characterization

^{125}I radiolabelling of CRCs was used to detect the efficacy of peptide loading onto collagen gels.

5.1.1 Loading of P-CRC versus CRC-RGDS to collagen scaffolds

The first experiment evaluated the efficiency of CRC binding to collagen scaffolds when additional bioactive amino acids were added to the peptides (CRC). For this study, one bioactive CRC (CRC-RGDS) was chosen randomly to be compared with P-CRC to evaluate the effect of incorporating four bioactive amino acids to the middle blocks of P-CRC. The binding efficiency of P-CRC was compared with that of CRC-RGDS in an experiment where different concentrations of peptide solutions were added to several identical disks of type I collagen scaffolds cut to the same size each, and left overnight. The following day, excess peptides were removed from the collagen surfaces by washing. The remaining peptides were quantified by radioactivity count. Figure 10 shows the results of this experiment.

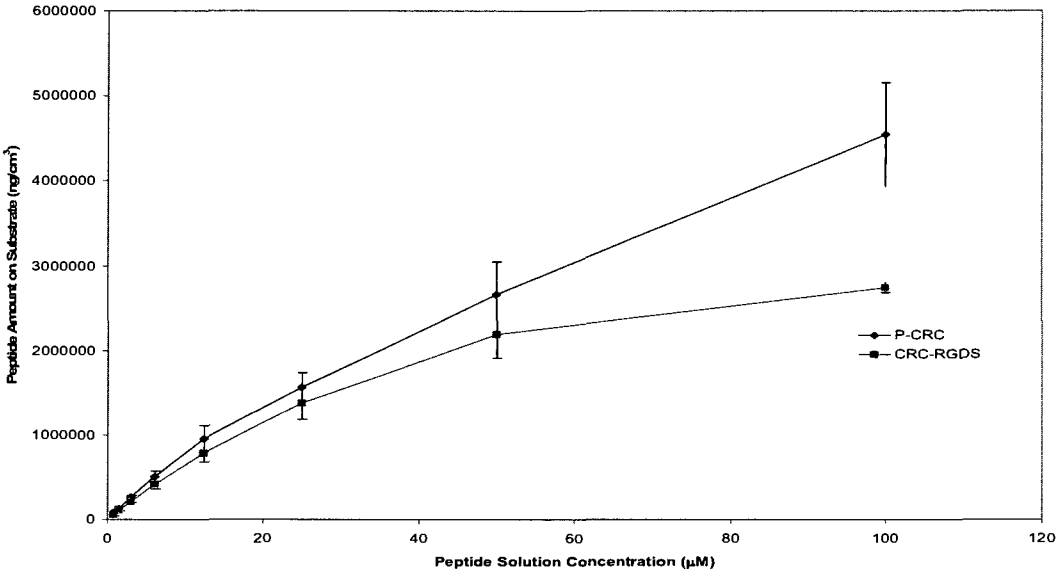

Figure 10: Remaining peptide on collagen scaffolds versus the initial peptide concentration in solution. P-CRC is compared to CRC-RGDS.

The amount of both peptides (P-CRC and CRC-RGDS) remaining on the collagen scaffolds inceases with their initial concentration in the solution. Also the amount of CRC-RGDS remaining on the scaffolds is almost the same as that of the P-CRC as both curves have similar trends. In addition a Two Way ANOVA (see Appendix C) test determined that the null hypothesis of equal means for both peptides can be accepted at a 0.05 level, hence showing that the quantity of peptides remaining on collagen surfaces is not significantly different between control (P-CRC) and modified peptide (CRC-RGDS). Since adding an additional bioactive sequence does not alter the whole CRC structure, only P-CRCs were used for all the other chemical surface characterization experiments.

5.1.2 Grafting of P-CRC with different linkers

As an alternative to adsorption, P-CRC peptides were also covalently attached to the surface of collagen scaffolds via crosslinking agents. Two different types of crosslinkers (EDC and Sulfo EGS) were used and compared for their efficiency in binding P-CRCs. In

the case of EDC, it was added to the collagen samples after the peptide was added. On the other hand, Sulfo EGS was added before the peptide was added to the scaffolds. Furthermore, a third test evaluated the efficiency of an activator (NHS) combined with EDC to improve the grafting rate of the peptide to the scaffold. Then once peptide was grafted, gels were washed for 2 day periods with a stringent washing buffer, 10% SDS-PBS, to simulate preparation steps that the samples undergo before being submitted for cell culturing. The results for these tests are as follows.

5.1.2.1 EDC/EDC-NHS/Sulfo EGS

Several tests were made varying the concentrations of EDC, NHS and Sulfo EGS to study differences amongst linkers and the activator. The results are shown in Figure 11.

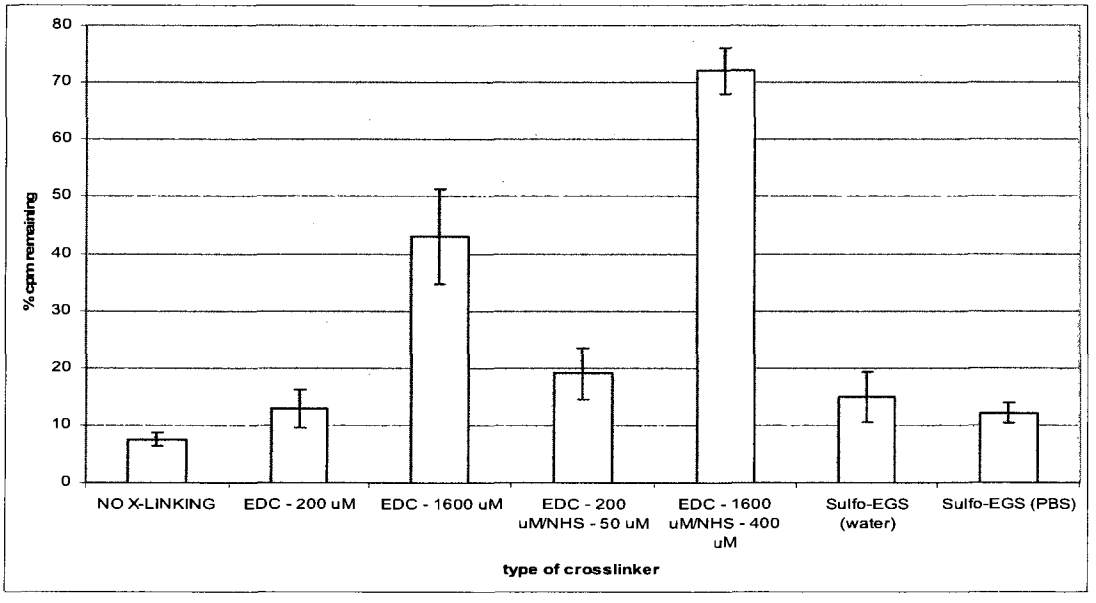

Figure 11: Percentage of peptide remaining on 10% collagen scaffolds at different crosslinker concentrations and type and after undergoing intense washing of gels after peptide grafting. P-CRC was evaluated in this study. In the case of Sulfo EGS, this crosslinker was dissolved in either water or PBS.

Despite the absence of crosslinker on the control sample, there were still the peptides remaining on the collagen surface. However the amount of the peptide was below the one found in all other samples in which a crosslinker was used.

The amount of the peptide remaining on the samples with EDC was increased with the amount of EDC added to the system: at higher EDC concentrations (1600 uM), more peptide was found on the scaffolds. Moreover, when NHS activator was used in conjunction with EDC there was more P-CRC peptide bound to the collagen scaffolds even after two days of washing with stringent buffer, 10% SDS-PBS.

Also more peptide was grafted to collagen with EDC (in both the presence and absence of NHS) than with Sulfo EGS solution of a similar concentration prepared in either water or PBS (1500uM). When Sulfo EGS used at a very high concentration of 1500uM, compared to EDC at a lower amount of 200uM with 50uM NHS, there was still less peptide binding than with the latter crosslinker.

Adding NHS to EDC of 1600 uM nearly doubled the amount of the peptide remaining on collagen surfaces.

A One Way ANOVA (see Appendix C) confirmed that the amount of peptide remaining on the collagen surfaces depended on the crosslinker used since the null hypothesis of equality of all samples was rejected at a significance level of 0.05.

5.1.2.2 Effect of adsorption/absorption

From the control sample it is clear that not all P-CRC molecules were covalently bound to the collagen surfaces. Some remained adsorbed to the collagen samples despite the washing process that they underwent for 2 days. Also some P-CRC molecules could have penetrated deeply into the collagen matrix by absorption, which could explain the presence of CRCs on the non covalently bound control despite intense washing that should have removed adsorbed P-CRC molecules.

5.1.2.3 Effect of 10% SDS-PBS washing of gels

The washing buffer clearly had an effect on the amount of peptide remaining on the collagen surface as in all samples including no crosslinking, there was never 100% peptides remaining on the surface after intense washing as indicated by Figure 11. This result indicated that by the time samples were ready for cell culturing, lots of peptides must have been lost.

5.1.3 Effects of 10% FBS washing of samples modified with P-CRC grafted at different EDC/NHS concentrations.

The effect of cell culture media on peptide retention on collagen surfaces was evaluated. P-CRC peptide was grafted onto collagen scaffolds via EDC or EDC/NHS linkers at different concentrations of both chemicals, and exposed to cell culture media for 336 hours.

The results of this experiment are depicted in Figure 12.

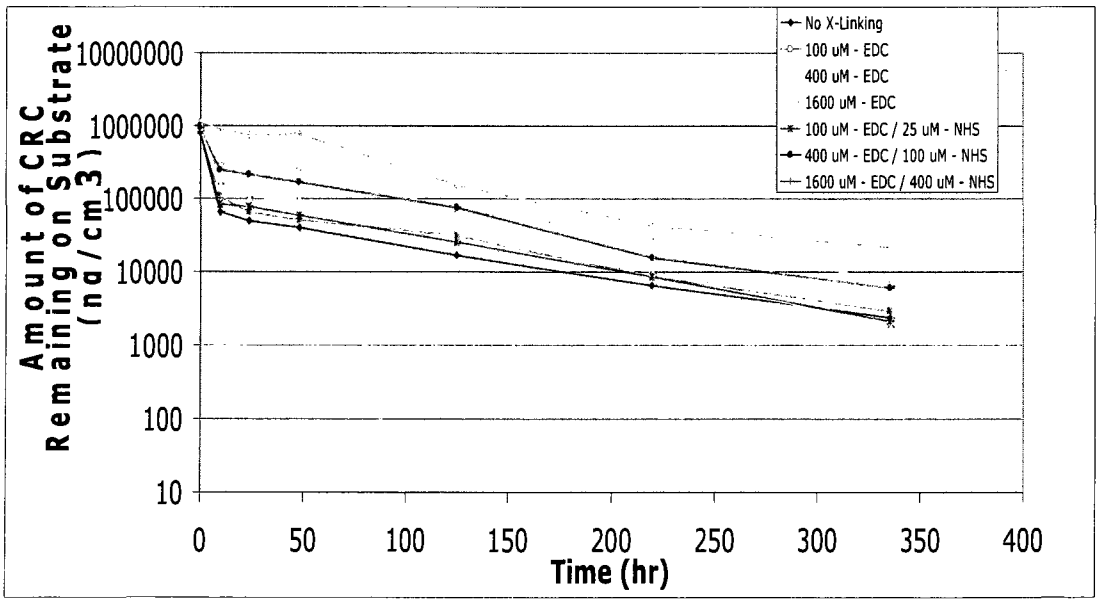

Figure 12: Amount of P-CRC peptide remaining on 10% collagen scaffolds at different EDC crosslinker concentration, with NHS activator and without it. Only P-CRC was evaluated in this study.

Peptide density on collagen scaffolds started at near 1000000 ng/cm^3 for all samples. However it decreased dramatically with longer exposure to the serum media. It fell to about 0.1% of its initial value.

In addition, samples where the highest concentrations of EDC and NHS were used for grafting, resulted in the highest amounts of peptide retained on collagen throughout the experiment. A Two Way ANOVA (see Appendix C) at 0.05 significance level, indicated that both time and EDC/NHS concentrations influence peptide retention density on collagen scaffolds as observed graphically.

5.2 Cell culturing results

MSC culturing on collagen scaffolds was always evaluated in two parts. The first part examined cell growth and proliferation efficiency and the second part focused on the cell differentiation into osteoblasts.

5.2.1 Cell growth and proliferation

MSCs were directly counted from the images taken during the 14 day study.

5.2.1.1 Morphology of MSC expansion on collagen scaffolds and tissue culture plate

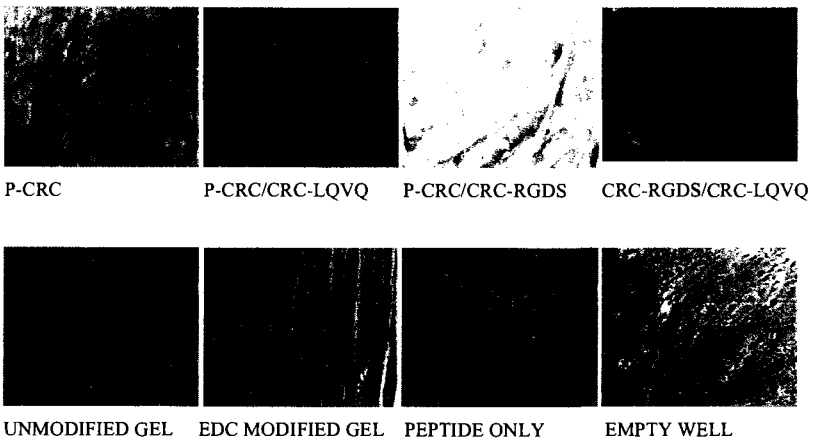

Figure 13: Images of MSCs cultured on 10% collagen scaffolds modified with CRC peptides and their controls. Pictures were taken on day 14 culture. Samples correspond to cells on P-CRC, P-CRC/CRC-LQVQ, P-CRC/CRC-RGDS, CRC-RGDS/CRC-LQVQ, Unmodified collagen scaffold, EDC modified collagen scaffold, Peptide only well (sample consisting of CRC-RGDS and CRC-LQVQ at an equal ratio) and Empty well. A 200X times magnification was used.

All samples from Figure 13, i.e. containing P-CRC, P-CRC/CRC-LQVQ, P-CRC/CRC-RGDS, and CRC-RGDS/CRC-LQVQ grafted to collagen scaffolds as well as the two controls containing unmodified collagen scaffolds and EDC modified collagen scaffolds had similar MSC growth patterns. Cells were widespread on the collagen. On P-CRC containing collagen scaffolds, cells appeared unhealthy compared to other samples containing CRC modified collagen. The other two controls consisting of the peptide only containing an equal mix of 1:1 (w/w) of CRC-RGDS/CRC-LQVQ and the other control that did not have either collagen scaffolds or peptides (empty well) appeared to have more cells than the rest of the samples. However on the peptide only well, cells appeared to be clustered in certain areas of the well.

5.2.1.2 Cell proliferation counts

Proliferation results of the MSCs on all samples were plotted in the histogram in Figure 14.

Cell proliferation numbers were very similar in all 4 types of CRC modified collagen samples and in the unmodified and EDC modified collagen control samples. Growth on these samples was also similar on all days although lower on first day of experiment, which is expected since cells mutliply after day 1 because they have the proper nutrients to do so. Also cell numbers hit a peak at about days 5-7 for these samples.

On the other hand, cell growth starts relatively low on day 1 of Peptides only sample and empty well control. Then they reach a large peak in cell growth around day 3 after which cell numbers dramatically decrease and then stabilize until the last day where cells start dying.

Cell proliferation appeared different depending on controls and samples. Next section will look at differentiation of MSCs on all controls and samples.

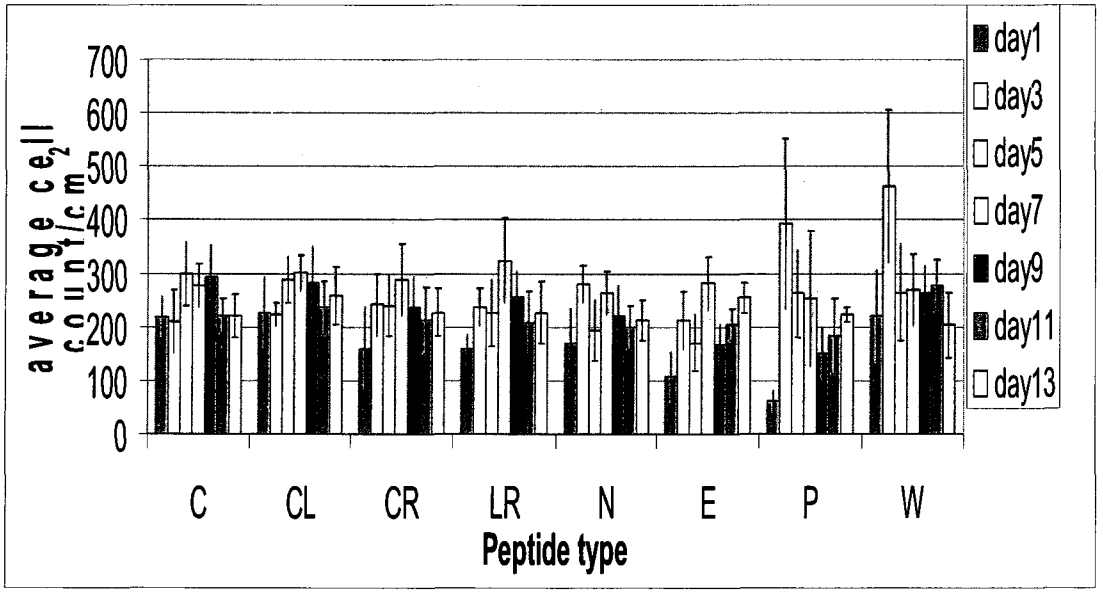

Figure 14: Average MSC count on the different collagen scaffolds and controls. C: P-CRC; CL: P-CRC/CRC-LQVQ; CR: P-CRC/CRC-RGDS; LR: CRC-LQVQ/CRC-RGDS; N: Unmodified collagen; E: EDC modified collagen; P: Peptides alone; W: Tissue culture plate well.

5.2.2 Differentiation of MSCs

MSCs were also differentiated on CRC modified collagen samples and controls during a 14 day study. Figure 15 shows pictures of the MSC differentiation experiment on CRC modified collagen scaffolds taken on day 14 of the test after alizarin red was added to stain for calcium deposits. Controls were not shown as they did not work.

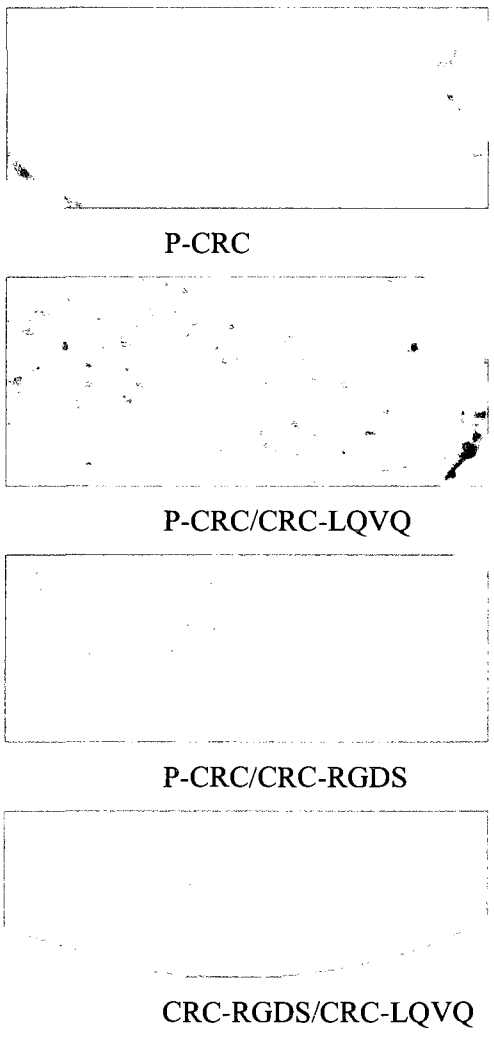

Figure 15: Alizarin red stained calcium deposits from MSCs cultures on P-CRC, P-CRC/CRC-RGDS, P-CRC/CRC-LQVQ and CRC-RGDS/CRC-LQVQ modified 10% collagen gels taken on day 14 of culture. A 200X times magnification was used.

Very rarely were calcium deposits observed on CRC-LQVQ/CRC-RGDS modified 10%collagen gels. Calcium staining however was observed on all other samples and mainly on the P-CRC/CRC-RGDS one. Cells on P-CRC only samples were not as healthy as ones on bioactive substrates.

Faint osteopontin staining was observed on all samples. P-CRC/CRC-RGDS samples had the most intense staining, indicating the presence of minimal to no osteopontin was found in the sample containing CRC-RGDS/CRC-LQVQ collagen. The same result was observed on the Peptide only control that contained a mixture of CRC-RGDS/CRC-LQVQ peptides.

Figure 16 shows pictures of this osteoblast differentiation done on day 14 of the whole cell culturing experiment.

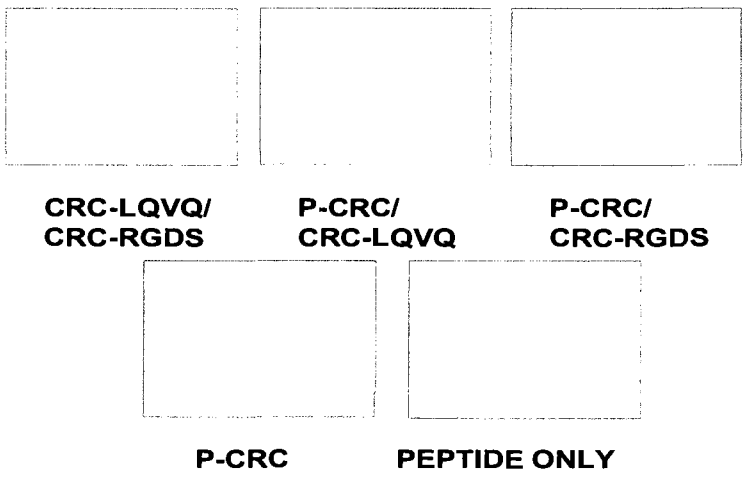

Figure 16: Osteopontin presence on CRC modified collagen samples and control cultured with MSCs that have differentiated into osteoblasts. Samples consisted respectively of P-CRC, P-CRC/CRC-LQVQ, P-CRC/CRC-RGDS and CRC-RGDS/CRC-LQVQ modified 10% collagen gels and Peptide only control containing CRC-RGDS/CRC-LQVQ only. Pictures depict day 14 of experiment. A 200X magnification was used.

Chapter 6: Discussion

6.1 Chemical Surface Modification

Plots of P-CRC and CRC-RGDS loading to the collagen scaffolds versus the amount of adsorbed peptide were similar to each other. The amount of peptide retained to the collagen scaffolds was comparable for both types of peptide as it grew with increasing concentration in solution. Hence both peptides attached to the scaffolds in a similar fashion.

Furthermore P-CRC and CRC-RGDS were shown to adsorb (possibly even absorb) to the collagen scaffolds at a statistically equal rate as indicated by a p-value of 0.37 for a significance level of α at 0.05. Hence, four amino acids to the CRC peptides are not long enough to alter the structural and mechanical properties of CRC, as observed previously. Fischer et al.[55] also found in a different study that P-CRC and CRC-RGDS have similar surface properties on glass substrates as they were found to adsorb similarly. Hence adding additional bioactive amino acids does not alter significantly the surface activities of the P-CRC peptide.

In addition, since now it has been seen that all CRC peptides tested in this project have similar loading properties; any differences found in cell culturing results will be attributed to other reasons than differences in loading efficiency amongst different peptides. Difference in cell culturing found among different CRCs is ascribed to the difference in action mechanism of each specific bioactive sequence on its own, i.e. the RGDS and LQVQ groups, or the lack of these amino acids.

Since the two different CRC peptides tested were found to behave similarly in terms of loading to the collagen scaffolds, only data for the P-CRC control was fitted to two different adsorption isotherms: the Langmuir and Freundlich models. Data fit better to the Freundlich model as indicated by a higher regression coefficient R^2 value of 0.9967 compared to a value of 0.9191 for the Langmuir adsorption isotherm. The mathematical equation obtained was thus as follows (refer to Appendix E):

$$q=8.5936C^{0.8486} \qquad \text{Equation 3}$$

Where k_F has a value of 8.5936 and n has a value of less than unity, *i.e.* 0.8486.
The adsorption constant k_F, which indicates the adsorption capacity of a material, is rather low in this case, indicating hence a smaller adsorption capacity of the collagen scaffolds for P-CRC. Also the adsorption affinity parameter n is also low as it is not unity, thus indicated a smaller affinity of the collagen for the CRCs.

6.1.1 Effect of different crosslinkers on grafting of P-CRC

P-CRC peptide was covalently grafted to collagen scaffolds by the means of different chemical linkers such as EDC, Sulfo EGS, and EDC with its activator NHS. A control consisting of loading peptides in the absence of any chemical linker was also prepared. The next section will discuss the results obtained for the washing of the samples with a stringent buffer after grafting of peptide.

6.1.1.1 Effect of adsorption/absorption

Figure 11 showed that even when no linker agent was added there was still some peptide being adsorbed onto the collagen scaffolds. This was also seen in the previous experiment when the control P-CRC peptide was compared to CRC-RGDS in a loading experiment using no crosslinker. However in this case, the measurements of radioactivity were taken after samples had been washed for 2 days in stringent buffer solutions. Hence any adsorbed peptide should have been washed away. Thus it is possible that some peptide had been initially adsorbed onto the surface of the collagen scaffold and then further absorbed deeper in the matrix of the control collagen samples. Therefore peptides were adsorbed, absorbed or covalently bound to the collagen scaffolds [60, 61].

6.1.1.2 Effect of 10% SDS-PBS washing of gels

The use of linkers favored the retention of peptides to the collagen scaffolds as can be seen in Figure 11 since more peptides were retained on the collagen scaffolds compared to the control of no linker. The amount of peptide retained in the no linker sample was 7.57% whereas this value grew up to about 13% with the lower linker amounts (i.e.200uM of EDC) and further increased to 72% with higher linker amounts (i.e.1600uM of EDC/400uM NHS). Peptides bound covalently to scaffolds were better at resisting the harsh conditions of the 10% SDS-PBS washing buffer.

In addition the higher the linker concentration the more peptide was retained to the surface after washing. The amount of peptide retained is proportional to the amount of EDC used. Hence as was shown above, at lower EDC concentrations, there was less peptide retained versus higher peptides grafted at higher EDC concentrations. Furthermore this amount was increased a lot more when activator particles, NHS, were used. Previous studies in which crosslinking of collagen fibers with either EDC alone or EDC/NHS were compared, found that using NHS promoted higher crosslinking rate as well as more crosslinking [41, 62] than in its absence. NHS contributed to the formation of a more stable N-acylisourea intermediate that resisted better to hydrolysis than the o-acylisourea intermediate formed by the EDC alone. A more stable intermediate encouraged more crosslinking of the collagen molecules.

Identical chemistries prevail in the crosslinking of collagen fibers to form collagen scaffolds as those of crosslinking a CRC peptide to the collagen scaffold surface; *i.e.* they both involve the attachment of a carboxylic acid group to an amine group [41]. Therefore EDC and NHS were found to have similar efficiencies on collagen crosslinking to the grafting of CRCs to collagen scaffold surfaces. Hence, better results of peptide grafting, when NHS was used, can be explained by a more stable intermediate formed after the EDC is added. Another study by Staros JV et al. [63] also found that grafting of peptides was improved significantly when EDC was used with NHS rather than EDC alone.

In contrast, the use of Sulfo EGS (at a concentration of 1500uM) did not lead to higher levels of peptide retention (14.99% and 12.09% for Sulfo EGS in water and in PBS respectively). The amount of peptides grafted with this crosslinker (at 1500 uM in both PBS

and water) was comparable to the amount grafted with 7.5 times lower amounts of the EDC linker (at 200 uM),.

Sulfo EGS targets randomly primary amines of the lysine residues [64] which can lead to strong intramolecular bonds within the CRC peptides which contain several chains of lysine residues. Such bonds could destabilize the CRC's ability to self assemble hence preventing the formation of a layer of peptides on the surface of the collagen scaffolds. Consequently it is possible that less peptide retained on surfaces when Sulfo EGS was used because CRCs were unable to form additional monolayers on the collagen surfaces.

On another note the presence of salts such as those coming from PBS buffers destabilizes Sulfo EGS structures rendering it less capable of performing its cross linking functions. Also P-CRC scaffold formation requires proper electrostatic interactions that are destabilized in the presence of salts [55]. Thereby leading to lower levels of peptide retention as compared to the value found when Sulfo EGS is dissolved in water.

For example the use of different types of linkers to crosslink diverse forms of RGDS peptides to a silicone scaffold, resulted in differences in the amount of peptides grafted to the surfaces of the biomaterial. These differences were attributed to the type of crosslinker used. [48]. Linkers that were more stable in water led to more peptides grafted. In the case of this experiment with CRC peptides, EDC with NHS seemed to be a more stable and an efficient combination of crosslinker with activator. For the rest of the experiments of this project (including the cell culturing ones), only EDC was used to graft CRCs to collagen scaffolds, since it was more efficient at grafting and less costly than Sulfo EGS. (NHS was not used in cell culturing experiments of this project, but will be tested further in a different study).

Despite the use of crosslinker, peptides can still come off the surfaces by hydrolysis of the bonds. Other peptides can come off by dissolution of the non specific bound peptides. [48] Hence grafting efficiency as well as hydrolysis of covalent bonds could explain the lower peptide retention percentages.

6.1.2 Grafting of P-CRC with different EDC/NHS concentrations and then washing in 10% FBS

Ultimately it was essential to test whether cell culturing conditions affected peptide retention to collagen scaffolds. Peptides were grafted at different EDC and NHS concentrations ranging from 100-1600uM of EDC and 0-400uM for NHS.

Once more Figure 12 showed that the use of NHS improved peptide retention to the collagen scaffolds as more peptides remained on collagen scaffolds modified with EDC and NHS as compared to EDC alone samples. What is more is the higher the EDC and NHS concentrations the more peptides are grafted as was seen in the previous tests under stringent washing conditions.

However as time passes, regardless of amount of crosslinker or activator used, the amount of P-CRC dropped drastically to about 10% of its original value in all samples. This last value could correspond to the covalently bound peptide and hence the efficiency of the grafting could be deduced to be only 10%. Further testing is still required to confirm this conclusion. In the meantime, several reasons will be given to explain the large peptide loss from the collagen scaffolds. For instance it is possible that peptides can still come off the surfaces by hydrolysis of the bonds. Other peptides can come off by dissolution of the non specific bound peptides as was seen earlier in one study [48].

Though hydrolysis of the bonds could be an option for the loss of peptides from the surface, it is more probable that the major loss is due mainly to the adsorbed CRCs that diffuse off the collagen scaffold surface. Also it is possible that peptides not only adsorbed to collagen but also absorbed to it. And peptides that also absorbed were diffused out as the peptide loss on collagen was gradual. When they were placed in a serum solution, peptides that were not covalently bound were washed off with time. Indeed a previous study found that CRC behavior was similar on glass substrates as it was on collagen substrates [55]. CRCs were displaced by serum proteins that were more affine to glass than CRCs and contributed to a decrease of these peptides to about 10% of their initial value as was found here. Also previously with the Freundlich model, the adsorption capacity constant K and the affinity constant n, were found to be relatively low indicating that the affinity of the substrate to the peptides seemed to be low which could explain why a lot of the adsorbed/absorbed peptide diffused out eventually from the collagen scaffolds.

To further this observation many studies [65, 66, 67, 68, 69] agree that adsorption of proteins to surfaces depends greatly on their affinity to the surface. This means that in the

presence of other proteins, such as serum proteins, there will be competition for the collagen surfaces. The diversity of proteins present in serum, could account for the presence of different affinity potentials, hence it is more likely to find serum proteins that are more affine to the collagen surface than the CRCs who have a lower K value. Thus peptides that have the highest affinity for the collagen will displace those which have the lowest affinity and replace them; such seemed to be the case of the CRC peptides in this experiment. Other parameters affecting competition amongst proteins adsorbing to surfaces are molecular weight, polarity and diffusion coefficient [70, 71].

Additionally, CRC peptides are formed by two leucine rich extremities that favor hydrophobic bonds as compared to the more prominently hydrophilic collagen type I molecule. Therefore it will be more likely that there are proteins amongst the serum used in this experiment that have better affinity for the collagen than CRCs, leading to the displacement of these latter ones by these more affine serum proteins.

Finally CRC peptides retained on collagen are most likely the CRCs that were grafted covalently on the collagen.

6.2 Cell Culturing

MSCs were cultured on modified collagen scaffolds with CRC peptides and on control samples consisting of non modified collagen, EDC modified collagen, unattached to collagen peptides (an equal mixture of CRC-RGDS/CRC-LQVQ) only, and on empty polystyrene surfaces. Collagen samples modified with CRC peptides consisted of CRC peptides with no bioactive sequence or P-CRC, an equal mixture of P-CRC and CRC-RGDS modified samples, an equal mixture of P-CRC and CRC-LQVQ modified samples and a final sample containing CRC-RGDS and CRC-LQVQ at a 1:1 ratio.

6.2.1 MSC proliferation on collagen scaffolds

MSC growth on collagen scaffolds and controls was monitored on a daily basis, however pictures were taken every other day. Despite being able to grow MSCs successfully, these cells were found to be very sensitive and hard to grow. Sterility of all solutions used for cell culturing was absolutely essential otherwise cells died immediately due to contamination.

The images of the MSCs on all collagen scaffolds modified with CRCs except the P-CRC containing sample, showed that cells had thin elongated shapes on the first day similarly to what another study observed when growing MSCs on silk scaffolds [8]. With time cells became bigger and had a round shape which was found to be attributable to ECM production [72]. On P-CRC modified collagen scaffolds cells looked unhealthy. Hence collagen modified with CRCs (except in the case of P-CRC alone) is a suitable material for growing healthy MSCs.

Cell proliferation was also evaluated as cells were counted every other day during the course of the experiment. Several patterns could be drawn for cell division on the different samples as cell growth appeared different according to sample or control estimated.

Indeed a two way ANOVA with a significance level α equal to 0.05 showed that all samples were not all equal in terms of cell proliferation (see Appendix D). Hence the modified collagen scaffolds with CRCs and control samples did not all have the same effect on the MSC growth.

To further investigate what treatments were not equal, a Tukey Scheffe test (see Appendix D) was also done on samples with a significance level α equal to 0.05. From this test it appeared that MSC proliferation on all collagen modified samples and plain collagen was equal. There are several possible explanations to these observations that will be examined next.

According to the Tukey Scheffe test, control samples that contained only the peptide mixture CRC-RGDS/CRC-LQVQ (non collagen bound peptides), and the empty control well (lacking CRC peptides and collagen scaffolds) came out as being statistically equal to each other for most days, i.e. for 4 out of 7 days counted. Hence cell adhesion, proliferation and growth did not seem to have much of an advantage on polystyrene plates when bioactive sequences RGDS and LQVQ from CRC were present. What is more is that the overall average number of cells on empty wells is slightly larger than on loose peptide only wells. A

possible explanation for this is that cells seemed to form clumps on the peptide only wells, which means that some cells were harder to identify and possibly the cell clusters were hiding some cells. If cells were not clearly seen, then they must have been erroneously omitted during cell count.

But another study by Maheshwari G. et al. 2000, [54], found that clustering of the peptides reduced the average ligand density required to allow proper NR6 cell migration and eventually spreading. It is possible that clustering of the CRC peptides on the polystyrene tissue culture plate may have inhibited proper cell dispersion on the well. Tightly located cells may have been unable to divide.

Also the CRC peptides present could have obstructed cell adhesion to plates if these former ones were unable to expose properly their bioactive sequences. And the large structures of the CRC peptides could have been blocking the surface of the plastic of the tissue culture plate (to which MSCs are known to bind properly [73]). Hence less MSCs were found in this sample.

Furthermore cell proliferation exhibited distinct patterns from sample to sample. Cells multiplied rapidly for the first few days of the experiment in the control samples containing only CRC peptides and in the empty control consisting of polystyrene tissue culture plate. MSCs were previously found to grow well on polystyrene dishes as was seen in this study [73]. Around day 5, cell proliferation decreased and seemed to reach a plateau until cells eventually started dying at about day 12. It appears that cells in those two control wells of the 48 well polystyrene plates, despite the slight differences described above, divided quickly at first when nutrients were abundant and space was not limited. Eventually cell density was too large to allow cell survival properly as cells did not have enough space and nutrients to favor cell division. Also it is to note that MSCs usually have an initial strong proliferation phase followed by an extracellular matrix (ECM) production phase that slows down cell division. [8]. Accumulation of ECM may have further limited mass transfer and oxygen dispersion in the well which could have contributed to a decline in cell proliferation in the latter days. [8; 74]

Cell proliferation followed a distinct pattern on all other samples *i.e* CRC and EDC modified collagen scaffolds. MSCs seemed to have a cell peak at later days, around days 5 to

7 when seeded in these samples. However these peaks were lower than for the unbound peptide and well only controls.

Unsuitable amounts of CRC peptides, that were unable to promote cell adhesion and proliferation, were possibly grafted to the collagen scaffolds. A study done before this one involving the CRC peptides, found that when RGDS was added to these peptides, human foreskin fibroblast cells, human umbilical vein endothelial cells, and rat neural stem cells grew better on culture dishes than in the absence of this bioactive sequence. Besides, rat neural stem cells proliferated more when more CRC-RGDS peptides were present. Indeed it was found that cell growth and proliferation was influenced mainly by the amount of CRC-RGDS present: the more peptide there was the more cells proliferated. In this project, the amount of CRCs used was kept constant at 1mg/ml in solution above scaffolds, as the main concern here was to investigate potential differences between the different bioactive sequences. [55]

The concentration at 1mg/ml used here was a standard concentration often used in literature [61, 75] but it is possible that this concentration is inadequate to promote better MSC proliferation and growth on collagen scaffolds. What's more a study reported that at a concentration of 1 mg/ml of RGDS peptides inhibited 70% and 40% retinal epithelial cell attachment to fibronectin and type I collagen respectively [76]. It is to note that collagen already contains RGDS groups as part of it chemical composition. It is probable that those endogenous RGDS peptides of collagen and the ones added in vitro result in an inappropriate density for proper MSC adhesion and proliferation on the scaffolds of this project.

Furthermore many studies found that there is an optimal amount for every scaffold-cell system of ligands that favor different cell activities including cell proliferation. Some of them have also found that if there was too much peptides the cells would not proliferate enough, while others found similar results with too little peptides being grafted. For instance in a study where the effect of RGDS, KQAGDV and VAPG ligands on biomaterial surfaces were evaluated for smooth muscle cells, it was found that at low concentration of ligand, cell migration and proliferation were better than at higher concentrations [77]. Other researchers found that murine melanoma cells best migrated on collagen scaffolds at intermediate levels of RGD peptides [78]. Fibroblasts were also found to have an optimal RGDS concentration above which cells proliferated less [79]. When there are too many ligands, it is possible that

cells are too tightly bound that they are unable to produce their own ECM which is necessary for their expansion. On the other hand, too little bonding or weak bonds results in cell death as cells need to bind through their integrin receptors to different molecules as this is their signaling medium that determines what these cells need to do. Also if they can not adhere they will not produce their ECM eventually and thus they will end up dying [11]. Therefore in this case where MSCs were grown on collagen scaffolds, the proper RGDS and LQVQ concentrations may have not been grafted leading to a decrease of cell division on collagen modified scaffolds. A test consisting of culturing MSCs on RGDS and LQVQ modified collagen scaffolds at various concentrations of these CRC peptides is still required to determine if there is an optimal concentration required for cell proliferation when using these peptides.

Another possibility is that the peptide had inadequate exposure of their bioactive sequences causing that their functions were not met. This option is very likely as the bioactive sequences, RGDS and LQVQ groups, both contain amino acids that have primary amines (i.e. the Arginine, R, and Glutamine, Q, residues). EDC crosslinkers work best on primary amines [41]. Consequently the R and Q residues are just as likely candidates for conjugation to the collagen surface as any other amino acid containing a primary amine group in CRC. Therefore if CRCs were bound via their RGDS and/or LQVQ groups to the collagen surfaces, the benefit of these bioactive sequences would have been lost. The primary objective of the peptides would have not been met leading to less cells adhering and proliferating on collagen surfaces.

In the case where CRCs were properly grafted to the collagen scaffolds i.e. not via their bioactive sequences, there is still another problem that could have hid the RGDS and LQVQ sequences, which is the very long spacer arms of CRCs. They could have obstructed the exposure of the unique bioactive sequences that they contain. Grafting of peptides does not guarantee to preserve the required structure of the entire peptide in order to allow this one to function as required. Some researchers found chemical grafting such as was done here with EDC, can alter the required peptide structure [80]. They opted instead for photolithography to bind their peptides. Both the crosslinker and the substrate onto which peptides must be grafted can affect the tridimensional structure of the peptide, which could

impede cell adhesion and proliferation as will be seen next. Cells might have had difficulty adhering to the bioactive sequences of CRCs.

Several studies found that the peptide microenvironment affected the way cells were bound to biomaterials and thus the ability of cells to bind, migrate and proliferate on these tissue engineered scaffolds. For instance peptide clustering was required for cell locomotion and further cell survival in a study that used YGRGD as a bioactive sequence for murine NR6 fibroblast culturing [54]. Houseman, B.T et *al.* (2001) found that longer molecules containing RGDS bioactive sequences had lower cell adhesion and proliferation since they believed that the larger molecules had lower chances of properly exposing their cell adhesion ligands[81]. What is more is that they found that the microenvironment onto which peptides were bound affected ligand exposure and cell adhesion.

The microenvironment can determine the binding degree of a peptide to a scaffold and how this one binds. Inadequately bound peptides can eventually come off. They can also take different shapes that are not the intended ones required for bioactive sequence exposure. CRC peptides are very long peptides (about 18 kDa) containing large spacer arms hosting only one bioactive sequence per peptide that can easily be hidden by the magnitude of this peptide.

Finally if indeed peptides have been grafted in sufficient amounts to promote cell adhesion, it is also possible that the bioactive sequences are not really appropriate for these types of cells in terms of adhesion and survival despite many studies used them with MSCs [82, 83]. It is also to note that cell numbers were not any better on CRC modified collagen scaffolds with bioactive sequences than the controls containing P-CRC, unmodified collagen and EDC modified collagen matrices. Every type of bioactive sequence is appropriate for different cells. For example a study found that REDV sequences were efficient for fibroblast adhesion yet not for smooth muscle cells [84]. A study by Sawyer et al. [85].concluded that RGD containing peptides were unable to promote MSC spreading which is required for cell survival. What is more is that other investigations found that there is a better peptide called P15 contained in type I collagen, that was found to be more efficient at promoting cell adhesion, proliferation and differentiation than RGDS [18]. Hence it would be interesting to reinforce the current type I collagen scaffolds with this P15 peptide for better MSC adhesion and survival or add the bioactive sequence of P15 to CRC peptides and see if indeed it was

the bioactive sequences chosen here that were inappropriate for MSCs or was it the CRCs that were improperly designed to expose these sequences. Further investigations are still required.

This section examined cell culturing results in terms of proliferation capacities of MSCs on CRC modified collagen scaffolds. The next part will look at the abilities of bioactive CRCs at promoting MSC osteogenesis.

6.2.2 MSC differentiation into osteoblasts

MSC differentiation into osteoblasts was also examined as the objective of this project was to regenerate bone tissue. The abilities of the peptide combinations P-CRC/CRC-RGDS, P-CRC/CRC-LQVQ and CRC-RGDS/CRC-LQVQ were looked at to see the osteogenesis capacities of each one. In summary, it was previously found that the peptide combination CRC-RGDS/CRC-LQVQ maintained neuronal stem cell state [reference]. In this study, the objective was to see whether this same peptide mixture was able to retain the stem cell state of the cells used in this experiment. To determine if osteogenesis had occurred, two markers were examined: the first used alizarin red to see whether calcium deposits had been formed as these are indicators of osteoblast formation. The second maker was osteopontin which is a linking protein secreted from bone cells. It plays a structural role as it constitutes an extracellular matrix protein secreted in bone tissue. Therefore the presence of these two markers indicated whether MSCs had differentiated into osteoblasts or not.

The results indicated after 14 days of culturing, that calcium deposits were present in P-CRC, P-CRC/CRC-RGDS and P-CRC/LQVQ collagen modified scaffolds. CRC-RGDS/CRC-LQVQ samples did not have any calcium deposits as seen in the images in Figure 15. Controls were not shown here as they were contaminated. Additionally, osteopontin was very mildly observed in all samples as depicted in Figure 16. Red coloring which indicates osteopontin presence is mostly abundant in the P-CRC/CRC-RGDS sample.

To explain these observations, it can be noted that cell culturing was only done for 2 weeks as opposed to 3 to 6 weeks in literature [86], because it was more expensive to culture

them for longer periods of time. Furthermore many listed papers stated that they observed mild calcium deposits at 2-3weeks, and that full osteoblastic phenotype could only be seen after at least 6 weeks of culturing. Differentiation of MSCs is a long process, as they first need to adhere to the surfaces, then proliferate, differentiate and release extracellular matrix, and finally mineralization of this matrix must occur. Hence it is a lengthy process that requires time that was not given to the MSCs in this experiment.

In a study comparing MSC osteogenesis on PLGA scaffolds versus PCL ones, found that it is possible that low MSC differentiation could have occurred because scaffolds were 2D and not 3D like reality. Here collagen scaffolds were also 2D and not 3D like reality hence possibly affecting cell development pathways [87].

Another group noted that hydrophobic surfaces better promoted osteogenesis of MSCs compared to hydrophilic ones [88]. Similar results were observed when MSCs were cultured on the more hydrophobic scaffolds, PLGA, which favored more osteogenesis than the more hydrophilic scaffolds PCL [87]. It was previously reported that type I collagen was better bound to hydrophilic surfaces compared to the hydrophobic ones [6]. Hence it is possible that the collagen scaffolds of this project were more hydrophilic because type I collagen was used, which in turn caused less osteoblast formation possibly because less MSCs were able to adhere to surfaces.

Possibly, the type of wettability of the surface altered the binding capacities of the diverse serum proteins and CRC peptides which in turn determined the types of ligands present on collagen scaffolds. Since cell proliferation and differentiation are dependent on the abilities of the cells for communication with its environment, if these ones lack the proper integrin receptors or are unable to see the adequate integrin binding elements required for adhesion, proliferation and osteogenesis, then cells will not become osteoblasts. This seems to have occured in this study. Also peptides were found previously not to retain much on the surfaces when placed in serum media for 336 hours. CRCs could have detached from the scaffold surfaces and floated in the environment; i.e. RGDS and LQVQ sequences are in solution rather than on the scaffolds. The importance of these peptides being bound onto collagen is evidenced by the fact that loose RGDS were found to inhibit MSC attachment in a previous study, which further hinders differentiation of MSCs [89]. And since a large amount of CRC peptides were suspsected to have been adsorbed to the surfaces, a large part

of them must have diffused out during the 2 weeks of cell culturing that the samples underwent. Many RGDS and LQVQ peptides could have been loosened in the cell culture media and hence inhibited cell adhesion and differentiation.

MSC binding elements and serum proteins that were indeed present on the scaffolds, could have affected integrin based cell signaling as cells could have been confused with the many different adhesion elements present on the surfaces as speculated in a previous paper [87]. Earlier it was reported that a variety of ligands (coming from serum proteins) including the CRC peptides were bound to the collagen scaffolds and possibly affected MSC binding. Consequently the presence of too many integrin binding elements could have caused that MSCs were confused as to what elements to bind to. Cell communication via cell signaling by integrin receptors is an essential factor that influences potentially cell proliferation and differentiation. [87]. To better illustrate each case of collagen modified scaffolds will be looked at next.

P-CRC/CRC-RGDS samples had the most calcium deposits and osteopontin presence due preferentially to the RGDS sequences. This sequence was found often in the literature to promote osteoblast formation of MSCs. Specific integrin receptors for the RGDS sequences must be present on the MSC surfaces which favored differentiation of MSCs [82]. However this differentiation was not very prominent possibly due to the presence of other signaling ligands from the serum proteins that caused confusion of the cells as to which path to undergo.

On the other hand, osteogenesis on P-CRC/CRC-LQVQ samples was even less evident as fewer calcium deposits were found and osteopontin markers were quite absent. It is possible that the presence of serum proteins and the LQVQ sequences also had the same effect of confusing cells onto which path to take. But since osteogenesis was even less important than the previous sample it could be that the LQVQ sequences are not very useful for osteogenesis. Indeed in the literature they have been often linked to cell adhesion and extensive proliferation in many cancerous cells but not much to osteogenesis. [90]. Furthermore since the level of calcium deposits and osteopontin markers was similar in this sample to the control containing P-CRC, it can be concluded that the LQVQ certainly did not have much effect on osteogenesis. Any signaling for osteoblast formation must have been from the serum proteins present in the medium.

In the case of the samples containing CRC-RGDS/CRC-LQVQ, the presence of the two different signaling molecules (as well as the ones from the serum proteins of the cell culture media that were also present in the other samples) could have caused similar confusion of the cells as explained above. More to this, it is possible that since RGDS containing samples favored osteogenesis and LQVQ ones did not as much, it could be hypothesized that there is some kind of dominance of one signaling system over the other. Probably, the effect of LQVQ was stronger than that of RGDS and hence instead of promoting osteogenesis, it maintained cells in stem cell stage.

In conclusion the low MSC proliferation and differentiation into osteoblasts on type I collagen scaffolds modified with CRC peptides were discussed in this section.

Chapter 7: Conclusions and Recommendations

MSC proliferation and osteogenic capacities on type I modified collagen scaffolds, with de novo synthesized CRC peptides containing bioactive ligands was examined. The peptides were composed of a control peptide P-CRC that lacked any bioactive sequence and the active CRCs that had one sequence each of the cell adhesion ligands from either fibronectin, RGDS which gave CRC-RGDS, or laminin LQVQ which gave CRC-LQVQ. Each collagen surface was modified by one or the combination of these peptides. The combination of peptides evaluated was: P-CRC, P-CRC/CRC-RGDS, P-CRC/CRC-LQVQ and CRC-LQVQ/CRC-RGDS.

Peptides were covalently grafted to the surfaces via EDC, EDC-NHS or Sulfo EGS chemistries for surface characterization with ^{125}I radiolabelling of the CRCs. It was found that EDC was the best chemical crosslinker as it favored more peptide binding to the surfaces. It was even more efficient when used with its activator NHS. Hence, this crosslinker was selected for further use in grafting peptides for cell culturing.

Peptides were found to potentially adsorb (and absorb) onto (and into) the collagen scaffolds. And a large amount of peptide loss resulted when samples were submerged in harsh washing buffers 10% SDS-PBS and serum culture media 10% FBS. Lost peptides were mainly attributed to the ones adsorb/absorb and hydrolysis of those improperly bound. Thus it still remains necessary to optimize the grafting process in order to prevent peptide loss by hydrolysis and to possibly increase the affinity of these peptides to their collagen substrate so that less are lost by diffusing out/off of the scaffold.

Proliferation of cells on these scaffolds were found to be rather weak compared to MSCs growth on the polystyrene surface of a 48 well plate tissue culture dish and on the surface of a peptide (CRC-RGDS/CRC-LQVQ) only coating in a 48 plate tissue culture dish used for cell culturing. The low cell numbers could be attributed to the possible steric hinderance of the cell adhesion ligands RGDS or LQVQ, or the inadequate amounts of CRCs with bioactive sequences present on the surfaces. Optimal CRC concentrations still remain to be determined. The peptides could be redesigned to better expose their bioactive sequences

and have better binding to the scaffolds, possibly by modification of the location of the bioactive sequences in the CRC peptide as that could favor better exposure of this group *eg.* moving the firm centre to a free end. Furthermore increasing the number of bioactive sequences in each CRC peptide could increase the chances of these sequences to be exposed for cell adhesion.

P-CRC/CRC-RGDS was the most promising peptide for promoting osteogenic differentiation. Other peptide combinations resulted in weak osteoblast formation. The CRC-RGDS/CRC-LQVQ combination blocked differentiation.

I have therefore shown that MSC differentiation into osteogenic cells can be influenced by modified, self-assembling peptides. Fibronectin derived RGDS was most promising in promoting differentiation, while a combination of LQVQ/RGDS was inhibiting. Further studies will be needed to be done to determine the efficacy of this peptide combination in modulating retention of stem cell properties in MSCs.

References

1. Gill, J. and Fenton, 2008, ''S. Preliminary Statistics on Transplantation and Waiting List: 2007 CORR Preliminary Report.'' http://secure.cihi.ca/cihiweb/en/downloads/2008-Tx_Wait_MT_persentation.ppt#436,20,Transplants1 and Waiting List at Year-end, 2 Canada, 1996-2007 [viewed September 8, 2008].

2. Kuo, C.K. & Tuan, R.S. 2003, "Tissue engineering with mesenchymal stem cells", *IEEE engineering in medicine and biology magazine : the quarterly magazine of the Engineering in Medicine & Biology Society*, vol. 22, no. 5, pp. 51-56.

3. Lee, W., Debasitis, J.C., Lee, V.K., Lee, J.H., Fischer, K., Edminster, K., Park, J.K. & Yoo, S.S. 2008, "Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication", *Biomaterials*, (2008).

4. McLaughlin, C.R., Fagerholm, P., Muzakare, L., Lagali, N., Forrester, J.V., Kuffova, L., Rafat, M.A., Liu, Y., Shinozaki, N., Vascotto, S.G., Munger, R. & Griffith, M. 2008, "Regeneration of corneal cells and nerves in an implanted collagen corneal substitute", *Cornea*, vol. 27, no. 5, pp. 580-589.

5. Curtis, M.W. and Russell, B.2009," Cardiac Tissue Engineering." *The Journal of Cardiovascular Nursing.*

6. Torun, G., Korkusuz, F., Korkusuz, P. and Hasirci, V. 2004, "In Vivo Tissue Engineering of Bone Using Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) and Collagen Scaffolds." *Tissue Engineering*, vol. 10, no. 7/8, pp. 1234-50.

7. Sykova, E., Jendelova, P., Urdzikova, L., Lesny, P. & Hejcl, A. 2006, "Bone marrow stem cells and polymer hydrogels--two strategies for spinal cord injury repair", *Cellular and molecular neurobiology*, vol. 26, no. 7-8, pp. 1113-1129.

8. Wang, Y., Kim, U.J., Blasioli, D.J., Kim, H.J. & Kaplan, D.L. 2005, "In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells", *Biomaterials*, vol. 26, no. 34, pp. 7082-7094.

9. Donzelli, E., Salvade, A., Mimo, P., Vigano, M., Morrone, M., Papagna, R., Carini, F., Zaopo, A., Miloso, M., Baldoni, M. & Tredici, G. 2007, "Mesenchymal stem cells cultured on a collagen scaffold: In vitro osteogenic differentiation", *Archives of Oral Biology*, vol. 52, no. 1, pp. 64-73.

10. Pountos, I., Jones, E., Tzioupis, C., McGonagle, D. & Giannoudis, P.V. 2006, "Growing bone and cartilage. The role of mesenchymal stem cells", *The Journal of bone and joint surgery.British volume*, vol. 88, no. 4, pp. 421-426.

11. Docheva, D., Popov, C., Mutschler, W. & Schieker, M. 2007, "Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system", *Journal of Cellular and Molecular Medicine*, vol. 11, no. 1, pp. 21-38.

12. Mori, M., Isobe, M., Yamazaki, Y., Ishihara, K. and Nakabayashi, N. 2000, "Restoration of segmental bone defects in rabbit radius by biodegradable capsules containing recombinant human bone morphogenetic protein-2." *Journal of Biomedical Material Research*, vol. 50, pp.191.

13. Bahr, W., Stricker, A., Gutwald, R. & Wellens, E. 1999, "Biodegradable osteosynthesis material for stabilization of midface fractures: experimental investigation in sheep", *Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery*, vol. 27, no. 1, pp. 51-57.

14. Habal, M.B. 1997, "A specialist's view of the new technology: the absorbable plating system", *The Journal of craniofacial surgery*, vol. 8, no. 2, pp. 83.

15. Pietrzak, W.S., Verstynen, M.L. & Sarver, D.R. 1997, "Bioabsorbable fixation devices: status for the craniomaxillofacial surgeon", *The Journal of craniofacial surgery*, vol. 8, no. 2, pp. 92-96.

16. Kadereit, S. 2005, "Adult Stem Cells." http://www.isscr.org/public/adultstemcells.htm [viewed September 8, 2008].

17. Schafer, R. & Northoff, H. 2008, "Cardioprotection and cardiac regeneration by mesenchymal stem cells", *Panminerva medica*, vol. 50, no. 1, pp. 31-39.

18. Yang, X.B., Bhatnagar, R.S., Li, S. & Oreffo, R.O. 2004, "Biomimetic collagen scaffolds for human bone cell growth and differentiation", *Tissue engineering*, vol. 10, no. 7-8, pp. 1148-1159.

19. Leonardi, E., Devescovi, V., Perut, F., Ciapetti, G. & Giunti, A. 2008, "Isolation, characterisation and osteogenic potential of human bone marrow stromal cells derived from the medullary cavity of the femur", *La Chirurgia degli organi di movimento*, vol. 92, no. 2, pp. 97-103.

20. Ciapetti, G., Ambrosio, L., Marletta, G., Baldini, N. & Giunti, A. 2006, "Human bone marrow stromal cells: In vitro expansion and differentiation for bone engineering", *Biomaterials*, vol. 27, no. 36, pp. 6150-6160.

21. Oliveira, J.M., Rodrigues, M.T., Silva, S.S., Malafaya, P.B., Gomes, M.E., Viegas, C.A., Dias, I.R., Azevedo, J.T., Mano, J.F. & Reis, R.L. 2006, "Novel hydroxyapatite/chitosan bilayered scaffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells", *Biomaterials*, vol. 27, no. 36, pp. 6123-6137.

22. Marolt, D., Augst, A., Freed, L.E., Vepari, C., Fajardo, R., Patel, N., Gray, M., Farley, M., Kaplan, D. & Vunjak-Novakovic, G. 2006, "Bone and cartilage tissue constructs grown using human bone marrow stromal cells, silk scaffolds and rotating bioreactors", *Biomaterials*, vol. 27, no. 36, pp. 6138-6149.

23. Meinel, L., Karageorgiou, V., Fajardo, R., Snyder, B., Shinde-Patil, V., Zichner, L., Kaplan, D., Langer, R. & Vunjak-Novakovic, G. 2004, "Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow", *Annals of Biomedical Engineering*, vol. 32, no. 1, pp. 112-122.

24. Byrne, E.M., Farrell, E., McMahon, L.A., Haugh, M.G., O'Brien, F.J., Campbell, V.A., Prendergast, P.J. & O'Connell, B.C. 2008, "Gene expression by marrow stromal cells in a porous collagen-glycosaminoglycan scaffold is affected by pore size and mechanical stimulation", *Journal of materials science.Materials in medicine*, vol. 19, no. 11, pp. 3455-3463.

25. Hulmes, D.J. 2002, "Building collagen molecules, fibrils, and suprafibrillar structures", *Journal of structural biology*, vol. 137, no. 1-2, pp. 2-10.

26. Hulmes, D.J. 1992, "The collagen superfamily--diverse structures and assemblies", *Essays in biochemistry*, vol. 27, pp. 49-67.

27. Orgel, J.P., Irving, T.C., Miller, A. & Wess, T.J. 2006, "Microfibrillar structure of type I collagen in situ", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 24, pp. 9001-9005.

28. Kadler, K.E., Holmes, D.F., Trotter, J.A. and Chapman, J.A. 1996," Collagen fibril formation", *Biochemical Journal,* vol. 316, no. 1, pp. 1–11.

29. Williams, B.R., Gelman, R.A., Poppke, D.C. and Piez, K.A.1978"Collagen fibril formation".*The Journal of Biological Chemistry*, vol. 253, no.18, pp. 6578.

30. Glowacki, J. 2007, "Impact of postmenopausal osteoporosis on the oral and maxillofacial surgery patient", *Oral and maxillofacial surgery clinics of North America*, vol. 19, no. 2, pp. 187-98, vi.

31. Bhatnagar, R.S., Qian, J.J. & Gough, C.A. 1997, "The role in cell binding of a beta-bend within the triple helical region in collagen alpha 1 (I) chain: structural and biological evidence for conformational tautomerism on fiber surface", *Journal of Biomolecular Structure & Dynamics*, vol. 14, no. 5, pp. 547-560.

32. Qian, J.J. & Bhatnagar, R.S. 1996, "Enhanced cell attachment to anorganic bone mineral in the presence of a synthetic peptide related to collagen", *Journal of Biomedical Materials Research*, vol. 31, no. 4, pp. 545-554.

33. Tsuchiya, K., Chen, G., Ushida, T., Matsuno, T. and Tateishi, T. 2001," Effects of cell adhesion molecules on adhesion of chondrocytes, ligament cells and mesenchymal stem cells". *Materials Science and Engineering C* vol.17, pp. 79-82.

34. Kundu, A.K. & Putnam, A.J. 2006, "Vitronectin and collagen I differentially regulate osteogenesis in mesenchymal stem cells", *Biochemical and biophysical research communications*, vol. 347, no. 1, pp. 347-357.

35. Mauney, J.R., Kirker-Head, C., Abrahamson, L., Gronowicz, G., Volloch, V. & Kaplan, D.L. 2006, "Matrix-mediated retention of in vitro osteogenic differentiation potential and in vivo bone-forming capacity by human adult bone marrow-derived mesenchymal stem cells during ex vivo expansion", *Journal of biomedical materials research.Part A*, vol. 79, no. 3, pp. 464-475.

36. Xiao, Y., Qian, H., Young, W.G. & Bartold, P.M. 2003, "Tissue engineering for bone regeneration using differentiated alveolar bone cells in collagen scaffolds", *Tissue engineering*, vol. 9, no. 6, pp. 1167-1177.

37. Yunoki, S. & Matsuda, T. 2008, "Simultaneous processing of fibril formation and cross-linking improves mechanical properties of collagen", *Biomacromolecules*, vol. 9, no. 3, pp. 879-885.

38. Charulatha, V. & Rajaram, A. 2003, "Influence of different crosslinking treatments on the physical properties of collagen membranes", Biomaterials, vol. 24, no. 5, pp. 759-767.

39. Ibusuki, S., Halbesma, G.J., Randolph, M.A., Redmond, R.W., Kochevar, I.E. & Gill, T.J. 2007, "Photochemically cross-linked collagen gels as three-dimensional scaffolds for tissue engineering", *Tissue engineering*, vol. 13, no. 8, pp. 1995-2001.

40. Wikipedia.2008, "Carbodiimide."http://en.wikipedia.org/wiki/Carbodiimide#EDC [viewed September 8, 2008].

41. Wikipedia. 2007, "N-hydroxysuccinimide". http://upload.wikimedia.org/wikipedia/en/6/6f/Nhs.png [viewed September 8, 2008].

42. Olde Damink, L.H., Dijkstra, P.J., van Luyn, M.J., van Wachem, P.B., Nieuwenhuis, P. & Feijen, J. 1996, "Cross-linking of dermal sheep collagen using a water-soluble carbodiimide", *Biomaterials*, vol. 17, no. 8, pp. 765-773.

43. Roach, P., Eglin, D., Rohde, K. & Perry, C.C. 2007, "Modern biomaterials: a review - bulk properties and implications of surface modifications", *Journal of materials science.Materials in medicine*, vol. 18, no. 7, pp. 1263-1277.

44. Biondi, M., Ungaro, F., Quaglia, F. & Netti, P.A. 2008, "Controlled drug delivery in tissue engineering", *Advanced Drug Delivery Reviews*, vol. 60, no. 2, pp. 229-242.

45. Weeks, B.S., Nomizu, M., Ramchandran, R.S., Yamada, Y. & Kleinman, H.K. 1998, "Laminin-1 and the RKRLQVQLSIRT laminin-1 alpha1 globular domain peptide stimulate matrix metalloproteinase secretion by PC12 cells", *Experimental cell research*, vol. 243, no. 2, pp. 375-382.

46. Nomizu, M., Kim, W.H., Yamamura, K., Utani, A., Song, S.Y., Otaka, A., Roller, P.P., Kleinman, H.K. & Yamada, Y. 1995, "Identification of cell binding sites in the laminin alpha 1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides", The *Journal of biological chemistry*, vol. 270, no. 35, pp. 20583-20590.

47. Cardarelli, P.M., Yamagata, S., Taguchi, I., Gorcsan, F., Chiang, S.L. & Lobl, T. 1992, "The collagen receptor alpha 2 beta 1, from MG-63 and HT1080 cells, interacts with a cyclic RGD peptide", *The Journal of biological chemistry*, vol. 267, no. 32, pp. 23159-23164.

48. Lateef, S.S., Boateng, S., Hartman, T.J., Crot, C.A., Russell, B. & Hanley, L. 2002, "GRGDSP peptide-bound silicone membranes withstand mechanical flexing in vitro and display enhanced fibroblast adhesion", *Biomaterials,* vol. 23, no. 15, pp. 3159-3168.

49. Massia, S.P. & Hubbell, J.A. 1991, "An RGD spacing of 440 nm is sufficient for integrin alpha V beta 3-mediated fibroblast spreading and 140 nm for focal contact and stress fiber formation", *The Journal of cell biology*, vol. 114, no. 5, pp. 1089-1100.

50. Aota, S., Nomizu, M. & Yamada, K.M. 1994, "The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function", *The Journal of biological chemistry*, vol. 269, no. 40, pp. 24756-24761.

51. Bhadriraju, K. & Hansen, L.K. 2000, "Hepatocyte adhesion, growth and differentiated function on RGD-containing proteins", *Biomaterials,* vol. 21, no. 3, pp. 267-272.

52. Garcia, A.J., Vega, M.D. & Boettiger, D. 1999, "Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation", *Molecular biology of the cell*, vol. 10, no. 3, pp. 785-798.

53. Mann, B.K. & West, J.L. 2002, "Cell adhesion peptides alter smooth muscle cell adhesion, proliferation, migration, and matrix protein synthesis on modified surfaces and in polymer scaffolds", *Journal of Biomedical Materials Research*, vol. 60, no. 1, pp. 86-93.

54. Maheshwari, G., Brown, G., Lauffenburger, D.A., Wells, A. & Griffith, L.G. 2000, "Cell adhesion and motility depend on nanoscale RGD clustering", *Journal of cell science*, vol. 113 (Pt 10), no. Pt 10, pp. 1677-1686.

55. Fischer, S.E., Liu, X., Mao, H.Q. & Harden, J.L. 2007, "Controlling cell adhesion to surfaces via associating bioactive triblock proteins", *Biomaterials,* vol. 28, no. 22, pp. 3325-3337.

56. Pierce Protein Research Products, 2009, "Sulfo-EGS (Ethylene glycol bis[sulfosuccinimidylsuccinate])".
http://www.piercenet.com/Products/Browse.cfm?fldID=02030260 [viewed September 8, 2008].

57. Chizzonite, R., Truitt, T., Podlaski, F.J., Wolitzky, A.G., Quinn, P.M., Nunes, P., Stern, A.S. & Gately, M.K. 1991, "IL-12: monoclonal antibodies specific for the 40-kDa subunit block receptor binding and biologic activity on activated human lymphoblasts", *Journal of immunology (Baltimore, Md.: 1950)*, vol. 147, no. 5, pp. 1548-1556.

58. Laidler, K.J., Meiser, J.H., Physical Chemistry, Boston: *Houghton Mifflin Company*, 1999.

59. Saez, A., Suarez, J.M., Aliaga, F. and Bo, R.M., 1994, "La utilizacion de los procedimientos de comparaciones multiples en la investigacion educative en España." *Revista de Investigación Educativa*, vol. 23, no. 1, pp 396-404

60. Xu, Q., Chen, X., Fu, B., Ye, Y., Yu, L., Wang, J., Li, W. and Qingli, C. 1999. "Integrin v 3–RGDS interaction mediates fibrin-induced morphological changes of glomerular endothelial cells". *Kidney International*, vol. 56, pp. 1413–1422.

61. Hadley, M.A., Weeks, B.S., Kleinman, H.K. & Dym, M. 1990, "Laminin promotes formation of cord-like structures by Sertoli cells in vitro", *Developmental biology*, vol. 140, no. 2, pp. 318-327.

62. Sinz, A. 2003, "Chemical cross-linking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes", *Journal of mass spectrometry : JMS,* vol. 38, no. 12, pp. 1225-1237.

63. Staros, J.V., Wright, R.W. and Swingle, D.M. 1986, "Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions." *Analytical Biochemistry*, vol. 156, pp. 220-222.

64. Evgeniy V. Petrotchenko‡, Vyacheslav K. Olkhovik§, and Christoph H. Borchers, 2005,"Isotopically Coded Cleavable Cross-linker for Studying Protein-Protein Interaction and Protein Complexes" *Molecular & Cellular Proteomics*, vol. 4, no.8, pp.1167-1179.

65. Yu, Y., Ying, P.Q. and Jin, G.2004," Competitive Adsorption between Bovine Serum Albumin and Collagen Observed by Atomic Force Microscope. "*Chinese Chemical Letters*, vol.15, no.12, pp.1465-1468.

66. Walker, A.K., Wu, Y., Timmons, R.B., Kinsel, G.R. & Nelson, K.D. 1999, "Effects of protein-surface interactions on protein ion signals in MALDI mass spectrometry", *Analytical Chemistry*, vol. 71, no. 1, pp. 268-272.

67. Oleschuk, R.D., Mccomb, M.E., Chow, A., Ens, W., Standing, K.G., Perreault, H., Marois, Y. & King, M. 2000, "Characterization of plasma proteins adsorbed onto biomaterials. By MALDI-TOFMS", *Biomaterials,* vol. 21, no. 16, pp. 1701-1710.

68. Schleuder, D., Hillenkamp, F. & Strupat, K. 1999, "IR-MALDI-mass analysis of electroblotted proteins directly from the membrane: comparison of different membranes, application to on-membrane digestion, and protein identification by database searching", *Analytical Chemistry*, vol. 71, no. 15, pp. 3238-3247.

69. Grinnell, F. & Feld, M.K. 1982, "Fibronectin adsorption on hydrophilic and hydrophobic surfaces detected by antibody binding and analyzed during cell adhesion in serum-containing medium", *The Journal of biological chemistry*, vol. 257, no. 9, pp. 4888-4893.

70. Kim, K.S. and Kang, S.H. 1998, "Adsorption Properties of Bovine Serum Albumin on Porous Polymer Microgels". *Korea Polymer Journal*, vol. 6, no. 3, pp. 235-240.

71. Salgın, S., Takac, S. and Ozdamar, T.H. 2006, "Adsorption of bovine serum albumin on polyether sulfone ultrafiltration membranes: Determination of interfacial interaction energy and effective diffusion coefficient".*Journal of Membrane Science*, vol. 278, pp. 251–260.

72. von der Mark, K., Gauss, V., von der Mark, H. & Muller, P. 1977, "Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture", *Nature*, vol. 267, no. 5611, pp. 531-532.

73. Qian, L. & Saltzman, W.M. 2004, "Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification", *Biomaterials*, vol. 25, no. 7-8, pp. 1331-1337.

74. Fromstein, J.D., Zandstra, P.W., Alperin, C., Rockwood, D., Rabolt, J.F. & Woodhouse, K.A. 2008, "Seeding bioreactor-produced embryonic stem cell-derived cardiomyocytes on different porous, degradable, polyurethane scaffolds reveals the effect of scaffold architecture on cell morphology", *Tissue engineering.Part A*, vol. 14, no. 3, pp. 369-378.

75. Yamada T, Shimada Y, Kikuchi M.1996, "Integrin-specific tissue-type plasminogen activator engineered by introduction of the Arg-Gly-Asp sequence." *Biochemical and Biophysiological Research Communications.* Vol 228, no.2, pp. 306-311.

76. Avery RL, Glaser BM.1986" Inhibition of retinal pigment epithelial cell attachment by a synthetic peptide derived from the cell-binding domain of fibronectin." *Archives of Ophthalmology.*Vol.104, no8, pp.1220-1222.

77. Mann, B.K. & West, J.L. 2002, "Cell adhesion peptides alter smooth muscle cell adhesion, proliferation, migration, and matrix protein synthesis on modified surfaces and in polymer scaffolds", *Journal of Biomedical Materials Research*, vol. 60, no. 1, pp. 86-93.

78. Burgess, B.T., Myles, J.L. & Dickinson, R.B. 2000, "Quantitative analysis of adhesion-mediated cell migration in three-dimensional gels of RGD-grafted collagen", *Annals of Biomedical Engineering*, vol. 28, no. 1, pp. 110-118.

79. Neff, J.A., Tresco, P.A. & Caldwell, K.D. 1999, "Surface modification for controlled studies of cell-ligand interactions", *Biomaterials,* vol. 20, no. 23-24, pp. 2377-2393.

80. Sebra, R.P., Reddy, S.K., Masters, K.S., Bowman, C.N. & Anseth, K.S. 2007, "Controlled polymerization chemistry to graft architectures that influence cell-material interactions", *Acta biomaterialia,* vol. 3, no. 2, pp. 151-161.

81. Houseman, B.T. & Mrksich, M. 2001, "The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion", *Biomaterials*, vol. 22, no. 9, pp. 943-955.

82. Okochi, M., Nomura, S., Kaga, C. & Honda, H. 2008, "Peptide array-based screening of human mesenchymal stem cell-adhesive peptides derived from fibronectin type III domain", *Biochemical and biophysical research communications*, vol. 371, no. 1, pp. 85-89.

83. Salinas, C.N., Cole, B.B., Kasko, A.M. & Anseth, K.S. 2007, "Chondrogenic differentiation potential of human mesenchymal stem cells photoencapsulated within poly(ethylene glycol)-arginine-glycine-aspartic acid-serine thiol-methacrylate mixed-mode networks", *Tissue engineering*, vol. 13, no. 5, pp. 1025-1034.

84. Hubbell, J.A., Massia, S.P., Desai, N.P. & Drumheller, P.D. 1991, "Endothelial cell-selective materials for tissue engineering in the vascular graft via a new receptor", *Bio/technology (Nature Publishing Company)*, vol. 9, no. 6, pp. 568-572.

85. Sawyer, A.A., Hennessy, K.M. & Bellis, S.L. 2007, "The effect of adsorbed serum proteins, RGD and proteoglycan-binding peptides on the adhesion of mesenchymal stem cells to hydroxyapatite", *Biomaterials*, vol. 28, no. 3, pp. 383-392.

86. Matsuo, A., Yamazaki, Y., Takase, C., Aoyagi, K. & Uchinuma, E. 2008, "Osteogenic potential of cryopreserved human bone marrow-derived mesenchymal stem cells cultured with autologous serum", *The Journal of craniofacial surgery*, vol. 19, no. 3, pp. 693-700.

87. Chastain, S.R., Kundu, A.K., Dhar, S., Calvert, J.W. & Putnam, A.J. 2006, "Adhesion of mesenchymal stem cells to polymer scaffolds occurs via distinct ECM ligands and controls their osteogenic differentiation", *Journal of biomedical materials research.Part A,* vol. 78, no. 1, pp. 73-85.

88. Lim, J.Y., Taylor, A.F., Li, Z., Vogler, E.A. & Donahue, H.J. 2005, "Integrin expression and osteopontin regulation in human fetal osteoblastic cells mediated by substratum surface characteristics", *Tissue engineering*, vol. 11, no. 1-2, pp. 19-29.

89. Lee JY, Choo JE, Choi YS, Shim IK, Lee SJ, Seol YJ, Chung CP, Park YJ. 2009, "Effect of immobilized cell-binding peptides on chitosan membranes for osteoblastic differentiation of mesenchymal stem cells." *Biotechnol Appl Biochem,* vol.52, no. 1, pp. 69-77.

90. Kim, W.H., Nomizu, M., Song, S.Y., Tanaka, K., Kuratomi, Y., Kleinman, H.K. & Yamada, Y. 1998, "Laminin-alpha1-chain sequence Leu-Gln-Val-Gln-Leu-Ser-Ile-Arg (LQVQLSIR) enhances murine melanoma cell metastases", *International journal of cancer.Journal International du Cancer*, vol. 77, no. 4, pp. 632-639.

Appendix A

CRC Protein Sequences

(Alpha helical domains underlined, bioactive peptides in bold print)

CRC

MRGSHHHHHHGSRLGHELAEHKKKLAQLKSELAALKKELAEWEMTSLY
RDPMGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAG
PEGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEG
ARMPTSELDYRRSSLGHELAEHKKKLAQLKSELAALKKELAEWE

CRC-RGDS

MRGSHHHHHHGSRLGHELAEHKKKLAQLKSELAALKKELAEWEMeTSLY
RDPMGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAG
PEG**RGDS**AGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAG
A
GPEGARMPTSELDYRRSSLGHELAEHKKKLAQLKSELAALKKELAEWE

CRC-LQVQ

MRGSHHHHHHGSRLGHELAEHKKKLAQLKSELAALKKELAEWEMTSLY
RDPMGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAG
PEG**LQVQLSIR**AGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGA
GAGAGPEGARMPTSELDYRRSSLGHELAEHKKKLAQLKSELAALKKELA
EWE

Appendix B

Table B1: MSC counts for days 1, 3, 5, 7, 9, 11 and 13. Results are depicted in number of surviving cells.

	C	CL	CR	LR	N	E	P	W
day1	18	9	18	11	9	5	4	15
	13	13	14	12	3	8	3	20
	14	7	9	10	10	12	2	9
	14	13	11	11	9	5	2	13
	12	20	8	8	11	7	6	9
	11	17	3	8	16	2	4	9
day3	13	17	16	7	20	13	21	17
	12	13	15	15	17	18	18	32
	10	16	20	13	17	14	17	32
	13	15	11	10	10	9	31	31
	9	11	9	19	15	14	22	32
	10	10	18	16	15	10	31	30
day5	14	15	14	11	8	8	17	15
	23	16	14	17	8	14	19	12
	13	22	21	14	10	9	15	13
	19	21	11	13	11	13	11	13
	15	18	13	8	10	9	18	20
	17	22	17	14	17	10	19	20
day7	16	20	15	26	20	15	12	21
	17	23	16	15	17	17	12	16
	13	19	21	21	16	15	16	16
	15	13	24	18	15	15	20	14
	17	18	13	16	13	16	21	12
	17	16	16	18	14	19	12	15
day11	12	17	10	14	18	14	10	17
	11	13	16	13	10	15	9	20
	16	12	14	15	10	15	10	16
	14	16	13	15	13	10	8	15
	9	17	12	12	15	12	14	18
	14	15	13	9	10	10	12	16
day9	19	14	12	14	14	9	8	12
	19	13	13	18	12	11	9	16
	18	18	16	13	11	7	9	16
	12	14	15	17	16	10	8	15
	17	16	18	19	11	11	9	15
	20	22	11	9	16	8	10	24
day13	14	17	14	17	9	17	13	14
	14	14	15	13	13	12	8	10
	10	14	11	14	16	15	12	10
	16	17	10	16	16	15	9	12
	13	15	13	13	13	14	13	14
	13	11	13	11	9	13	23	11

Table B2: Two Way Anova results that tested the effect of day and CRC peptide and interaction between day and type of peptide on MSC proliferation grown on 10% collagen scaffolds. Tests were done by Microsoft Excel Software.

SUMMARY	C	CL	CR	LR	N	E	P	W	Total
Day1									
Count	6	6	6	6	6	6	6	6	48
Sum	82	79	63	60	58	39	21	75	477
Average	13.66667	13.16667	10.5	10	9.666667	6.5	3.5	12.5	9.9375
Variance	5.866667	23.36667	26.7	2.8	17.46667	11.5	2.3	19.9	22.48537
Day3									
Count	6	6	6	6	6	6	6	6	48
Sum	67	82	89	80	94	78	140	174	804
Average	11.16667	13.66667	14.83333	13.33333	15.66667	13	23.33333	29	16.75
Variance	2.966667	7.866667	17.36667	18.66667	11.06667	10.4	38.66667	35.2	48.91489
Day5									
Count	6	6	6	6	6	6	6	6	48
Sum	101	114	90	77	64	63	99	93	701
Average	16.83333	19	15	12.83333	10.66667	10.5	16.5	15.5	14.60417
Variance	13.76667	9.6	12.4	9.366667	11.06667	5.9	9.5	13.1	17.22296
Day7									
Count	6	6	6	6	6	6	6	6	48
Sum	95	109	105	114	95	97	93	94	802
Average	15.83333	18.16667	17.5	19	15.83333	16.16667	15.5	15.66667	16.70833
Variance	2.566667	11.76667	17.1	16	6.166667	2.566667	17.5	9.066667	10.38121
day11									
Count	6	6	6	6	6	6	6	6	48
Sum	76	90	78	78	76	76	63	102	639
Average	12.66667	15	13	13	12.66667	12.66667	10.5	17	13.3125
Variance	6.266667	4.4	4	5.2	11.06667	5.466667	4.7	3.2	8.006649
day13									
Count	6	6	6	6	6	6	6	6	48
Sum	80	88	76	84	76	86	78	71	639
Average	13.33333	14.66667	12.66667	14	12.66667	14.33333	13	11.83333	13.3125
Variance	3.866667	5.066667	3.466667	4.8	9.866667	3.066667	28.4	3.366667	7.410904
Day9									
Count	6	6	6	6	6	6	6	6	48
Sum	105	97	85	90	80	56	53	98	664
Average	17.5	16.16667	14.16667	15	13.33333	9.333333	8.833333	16.33333	13.83333
Variance	8.3	11.36667	6.966667	14	5.466667	2.666667	0.566667	16.26667	16.1844

Total								
Count	42	42	42	42	42	42	42	42
Sum	606	659	586	583	543	495	547	707
Average	14.42857	15.69048	13.95238	13.88095	12.92857	11.78571	13.02381	16.83333
Variance	10.10453	13.24332	14.97329	15.1806	13.48258	14.3676	47.38966	40.77642

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	1570.06	6	261.6766	24.7811	1.57E-23	2.131028
Columns	758.3214	7	108.3316	10.25914	1.91E-11	2.042358
Interaction	2423.512	42	57.70266	5.464514	8.19E-19	1.428234
Within	2956.667	280	10.55952			
Total	7708.56	335				

Table B3: Summary of One Way Anova done for days 1, 3, 5, 7, 9, 11, 13 with Microsoft Excel Software.

treatment	**C**	**CL**	**CR**	**LR**	**N**	**E**	**P**	**W**
day								
	TREATMENTS ARE NOT ALL EQUAL AT 95%							
1	CONFIDENCE							
	TREATMENTS ARE NOT ALL EQUAL AT 95%							
3	CONFIDENCE							
	TREATMENTS ARE NOT ALL EQUAL AT 95%							
5	CONFIDENCE							
7	ALL TREATMENTS ARE EQUAL AT 95% CONFIDENCE							
	TREATMENTS ARE NOT ALL EQUAL AT 95%							
9	CONFIDENCE							
	TREATMENTS ARE NOT ALL EQUAL AT 95%							
11	CONFIDENCE							
13	ALL TREATMENTS ARE EQUAL AT 95% CONFIDENCE							

Appendix C

Section 1. Raw data for P-CRC and CRC-RGDS loading to 10% collagen scaffolds

Table C 1: Data for stock solutions used in loading of P-CRC and CRC-RGDS on 10% collagen scaffolds.

Stock Solutions	
background cpm	99

100 μM P-CRC in 10 mM phosphate, pH7.5

Dilution	Count 1 (cpm)	Count 1 Corrected(cpm)	Count 2 (cpm)	Count 2 Corrected(cpm)	Count 3 (cpm)	Count 3 Corrected (cpm)	Avg (cpm)	Stdev (cpm)	actual cpm/mL	actual cpm/mg
1	235186	235087	241465	241366	238404	238305	238253	3140	2382527	1330872

100 μM CRC-RGDS in 10 mM phosphate, pH7.5

Dilution	Count 1 (cpm)	Count 1 Corrected(cpm)	Count 2 (cpm)	Count 2 Corrected(cpm)	Count 3 (cpm)	Count 3 Corrected (cpm)	Avg (cpm)	Stdev (cpm)	actual cpm/mL	actual cpm/mg
1	174537	174438	175858	175759	176208	176109	175435	881	1754353	957721

Table C 2: Data for radioactive counts per minute obtained after loading of P-CRC on 10% collagen scaffolds

P-CRC in 10 mM phosphate, pH 7.5												
cpm/mg for stock solution:			1330 872									
Protein Concentration (μM)	**Count 1 (cpm)**	**Count 1 Corrected (cpm)**	**Amount (μg/cm³)**	**Count 2 (cpm)**	**Count 2 Corrected (cpm)**	**Amount (μg/cm³)**	**Count 3 (cpm)**	**Count 3 Corrected(cpm)**	**Amount (μg/cm³)**	**Avg (ng/cm³)**	**Stdev(ng/cm³)**	**Stderror(ng/cm³)**
100	67554	67455	4181	67990	67891	4208	84897	84798	5256	4548000	613	354
50	47756	47657	2954	45516	45417	2815	36006	35907	2225	2665000	387	223
25	22846	22747	1410	28343	28244	1751	24968	24869	1541	1567000	172	99
12.5	17755	17656	1094	12699	12600	781	16111	16012	992	956000	160	92
6.25	9419	9320	578	7466	7367	457	8074	7975	494	509000	62	36
3.125	4454	4355	270	4160	4061	252	4704	4605	285	269000	17	10
1.5625	1703	1604	99	2211	2112	131	2522	2423	150	127000	26	15
0.78125	1226	1127	70	1622	1523	94	1333	1234	76	80000	13	7

Amount of protein added to tube (ug)	Adsorption efficiency	Protein Concentration (uM)
1343	**0.010**	**100**
671	**0.012**	**50**
336	**0.014**	**25**
168	**0.017**	**12.5**
84	**0.018**	**6.25**
42	**0.019**	**3.125**
21	**0.018**	**1.5625**
10	**0.023**	**0.78125**

Table C3: Data for radioactive counts per minute obtained after loading of CRC-RGDS on 10% collagen scaffolds

CRC-RGDS in 10 mM phosphate, pH 7.5												
cpm/mg for stock solution			957721									
Protein Concentration (μM)	Count 1 (cpm)	Count 1 Corrected (cpm)	Amount (μg/cm^3)	Count 2 (cpm)	Count 2 Corrected (cpm)	Amount (μg/cm^3)	Count 3 (cpm)	Count 3 Corrected (cpm)	Amount (μg/cm^3)	Avg(ng/cm^3)	stdev(ng/cm^3)	stderror(ng/cm^3)
100	32794	32695	2816	31650	31551	2717	31537	31438	2708	2747000	60	35
50	25449	25350	2183	22420	22321	1922	28891	28792	2480	2195000	279	161
25	18788	18689	1610	15233	15134	1303	14455	14356	1236	1383000	199	115
12.5	10286	10187	877	9444	9345	805	7944	7845	676	786000	102	59
6.25	5608	5509	474	4706	4607	397	4488	4389	378	416000	51	30
3.125	2405	2306	199	2709	2610	225	2659	2560	220	215000	14	8
1.5625	1274	1175	101	1427	1328	114	1393	1294	111	109000	7	4
0.78125	630	531	46	882	783	67	777	678	58	57000	11	6

Amount of protein added to tube (ug)	Adsorption efficiency	Protein Concentration (uM)
1374	**0.00600**	**100**
687	**0.010**	**50**
343	**0.012**	**25**
172	**0.014**	**12.5**
86	**0.015**	**6.25**
43	**0.015**	**3.125**
21	**0.015**	**1.5625**
11	**0.016**	**0.78125**

Note: Calculations for these previously presented tables will be presented with those for Serum stability experimental data later.

Statistical Analysis of data of P-CRC and CRC-RGDS loading to 10% collagen scaffolds. Summary of Two Way Anova results.

Table C 4: Summary of adsorption efficiency of CRC peptides (expressed in terms of fractions) used in Anova studies of P-CRC and CRC-RGDS loading to 10% collagen scaffolds experiments.

	P-CRC	CRC-RGDS
Protein Concentration (uM)	**Adsorption efficiency**	**Adsorption efficiency**
100	0.010162099	0.599839819
50	0.011907755	0.009586868
25	0.014007077	0.012081365
12.5	0.017086309	0.013730112
6.25	0.01821412	0.014548091
3.125	0.019233361	0.01499645
1.5625	0.018133615	0.015232164
0.78125	0.022940293	0.015972408

Table C5: Summary of count averages and other data used in Anova studies of P-CRC and CRC-RGDS loading to 10% collagen scaffolds experiments.

Protein Concentration (uM)	*Count*	*Sum*	*Average*	*Variance*
100	2	0.610002	0.305001	0.17386
50	2	0.021495	0.010747	2.69E-06
25	2	0.026088	0.013044	1.85E-06
12.5	2	0.030816	0.015408	5.63E-06
6.25	2	0.032762	0.016381	6.72E-06
3.125	2	0.03423	0.017115	8.98E-06
1.5625	2	0.033366	0.016683	4.21E-06
0.78125	2	0.038913	0.019456	2.43E-05
P-CRC	8	0.131685	0.016461	1.75E-05
CRC-RGDS	8	0.695987	0.086998	0.042944

Table C 6 : Table of Two Way Anova results obtained for the P-CRC and CRC-RGDS loading experiment done on 10% collagen scaffolds at a 0.05 significance level that test the equality of loading abilities of both types of CRC peptides, the effect of concentrations of peptide in solution and the interaction between concentrations and type of peptide.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.146719	7	0.02096	0.952644	0.524691	3.787044
Columns	0.019902	1	0.019902	0.904582	0.373232	5.591448
Error	0.154012	7	0.022002			
Total	0.320633	15				

Section 2. Raw data for P-CRC grafting with different crosslinkers and further washing in 10% SBS-PBS

Table C 7 : Raw data for P-CRC stock solutions prepared for the experiment involving grafting of peptide with different crosslinkers and further washing in 10% SBS-PBS

Stock Solutions									
background cpm	99								
50 uM CRC in 10 mM phosphate, pH7.5 (6/27/08)									
BEFORE									
Count 1 (cpm)	**Count 1 Corrected(cpm)**	**Count 2(cpm)**	**Count 2 Corrected(cpm)**	**Count 3(cpm)**	**Count 3 Corrected(cpm)**	**Avg(cpm)**	**Stdev(cpm)**	**actual cpm/mL**	**actual cpm/mg**
165698	165599	165323	165224	163306	163207	164676.6667	1286.505	1646766.667	1839757.197
AFTER									
Count 1 (cpm)	**Count 1 Corrected(cpm)**	**Count 2(cpm)**	**Count 2 Corrected(cpm)**	**Count 3(cpm)**	**Count 3 Corrected(cpm)**	**Avg(cpm)**	**Stdev(cpm)**	**actual cpm/mL**	**actual cpm/mg**
160961	160862	161011	160912	160207	160108	160627.3333	450.4501	1606273.333	1794518.303
50 uM CRC in 10 mM phosphate, pH7.5 (6/30/08)									
BEFORE									
Count 1 (cpm)	**Count 1 Corrected(cpm)**	**Count 2(cpm)**	**Count 2 Corrected(cpm)**	**Count 3(cpm)**	**Count 3 Corrected(cpm)**	**Avg(cpm)**	**Stdev(cpm)**	**actual cpm/mL**	**actual cpm/mg**
163125	163026	165242	165143	162645	162546	163571.6667	1381.816	1635716.667	1827412.207
AFTER									
Count 1 (cpm)	**Count 1 Corrected (cpm)**	**Count 2(cpm)**	**Count 2 Corrected (cpm)**	**Count 3(cpm)**	**Count 3 Corrected(cpm)**	**Avg(cpm)**	**Stdev (cpm)**	**actual cpm/mL**	**actual cpm/mg**
160739	160640	162003	161904	159608	159509	160684.3333	1198.115	1606843.333	1795155.104

Table C 8: : Before and after washing with 10% SDS PBS raw data, for P-CRC grafting on 10% collagen scaffolds using different crosslinkers.

NO X-LINKING						
Before cpm/mg	1839757		**after cpm/mg**	1794518		
BEFORE			**AFTER**			
Counts (cpm)	**Counts Corrected (cpm)**	**Amount (ng)**	**Counts (cpm)**	**Counts Corrected (cpm)**	**Amount (ng)**	**% Remaining**
13214	13115	7128	863	764	426	5.972
11599	11500	6251	936	837	466	7.462
7641	7542	4099	658	559	312	7.599
11239	11140	6055	857	758	422	6.976
9989	9890	5376	1004	905	504	9.381
6069	5970	3245	569	470	262	8.071
					AVG (ng)	7.577
					STDEV (ng)	1.136
					STDERROR (ng)	0.656
EDC - 200 uM						
Before cpm/mg	1839757		**after cpm/mg**	1794518		
BEFORE			**AFTER**			
Counts (cpm)	**Counts Corrected (cpm)**	**Amount (ng)**	**Counts (cpm)**	**Counts Corrected (cpm)**	**Amount (ng)**	**% Remaining**
13739	13640	7414	1573	1474	821	11.079
12611	12512	6801	1431	1332	742	10.914
8956	8857	4814	1399	1300	724	15.049
10600	10501	5708	1113	1014	565	9.900
8756	8657	4705				
7611	7512	4083	1401	1302	726	17.769
					AVG (ng)	12.942
					STDEV (ng)	3.338
					STDERROR (ng)	1.927
EDC - 1600 uM						
Before cpm/mg	1839757		**after cpm/mg**	1794518		
BEFORE			**AFTER**			

Counts (cpm)	Counts Corrected (cpm)	Amount (ng)	Counts (cpm)	Counts Corrected (cpm)	Amount (ng)	% Remaining
8089	7990	4343	2817	2718	1515	34.875
7184	7085	3851	3110	3011	1678	43.573
4554	4455	2422	2257	2158	1203	49.661
9001	8902	4838	2999	2900	1616	33.400
6447	6348	3450	2711	2612	1456	42.187
5331	5232	2844	2896	2797	1559	54.807
					AVG (ng)	43.084
					STDEV (ng)	8.281
					STDERROR (ng)	4.781

EDC - 200 uM/NHS - 50 uM						
before cpm/mg	1839757		after cpm/mg	1794518		
BEFORE			AFTER			
Counts (cpm)	Counts Corrected (cpm)	Amount (ng)	Counts (cpm)	Counts Corrected (cpm)	Amount (ng)	% Remaining
8787	8688	4722	2283	2184	1217	25.772
5025	4926	2677	964	865	482	18.004
4504	4405	2394	973	874	487	20.344
10527	10428	5668	1530	1431	797	14.069
5857	5758	3129	918	819	456	14.584
5913	5814	3160	1322	1223	682	21.566
					AVG (ng)	19.056
					STDEV (ng)	4.451
					STDERROR (ng)	2.570

EDC - 1600 uM/NHS - 400 uM						
Before cpm/mg	1839757		after cpm/mg	1794518		
BEFORE			AFTER			
Counts (cpm)	Counts Corrected (cpm)	Amount (ng)	Counts (cpm)	Counts Corrected (cpm)	Amount (ng)	% Remaining
11988	11889	6462	8149	8050	4486	69.417
7037	6938	3771	4829	4730	2636	69.894
6160	6061	3294	4478	4379	2440	74.070
8383	8284	4502	5622	5523	3078	68.356
5098	4999	2717	3569	3470	1934	71.171

5870	5771	3137	4560	4461	2486	79.256
					AVG (ng)	72.027
					STDEV (ng)	4.050
					STDERROR (ng)	2.338
Sulfo-EGS (water)						
Before cpm/mg	1827412		**after cpm/mg**	1795155		
BEFORE			**AFTER**			
Counts (cpm)	**Counts Corrected (cpm)**	**Amount (ng)**	**Counts (cpm)**	**Counts Corrected (cpm)**	**Amount (ng)**	**% Remaining**
19415	19316	10570	3378	3279	1827	17.281
12700	12601	6895	1714	1615	900	13.047
8829	8730	4777	1002	903	503	10.530
14844	14745	8069	1920	1821	1014	12.572
9373	9274	5075				
5431	5332	2918	1228	1129	629	21.557
					AVG (ng)	14.997
					STDEV (ng)	4.413
					STDERROR (ng)	2.548
Sulfo-EGS (PBS)						
Before cpm/mg	1827412		**After cpm/mg**	1795155		
BEFORE			**AFTER**			
Counts (cpm)	**Counts Corrected (cpm)**	**Amount (ng)**	**Counts (cpm)**	**Counts Corrected (cpm)**	**Amount (ng)**	**% Remaining**
15863	15764	8626	1797	1698	946	10.965
8239	8140	4454	911	812	452	10.155
4930	4831	2644				
19025	18926	10357	2845	2746	1530	14.770
12943	12844	7028	1635	1536	856	12.174
7020	6921	3787	942	843	470	12.400
					AVG (ng)	12.093
					STDEV (ng)	1.753
					STDERROR (ng)	1.012

Statistical Analysis for samples containing P-CRC grafted onto 10% collagen scaffolds with different crosslinkers and tested for stability in 10% SDS-PBS. A One Way Anova was used to verify non equality of the effect of the different crosslinkers used.

Table C9: Raw data used for statistical analysis of inequality of crosslinkers used when P-CRC was grafted on 10% collagen scaffolds and further washed with 10% SDS-PBS.

Data for Table	Average (ng)
NO X-LINKING	7.577
EDC - 200 μM	12.942
EDC - 1600 μM	43.084
EDC – 200 μM/NHS – 50 μM	19.056
EDC – 1600 μM/NHS - 400 μM	72.027
Sulfo-EGS (water)	14.997
Sulfo-EGS (PBS)	12.093

Table C 10: Summary of percentage of P-CRC remaining on 10% collagen scaffolds following crosslinking with different chemicals. Data used in statistical analysis.

Peptide % Remaining						
NO X-LINKING	EDC - 200 μM	EDC - 1600 μM	EDC - 200 μM/ NHS - 50 μM	EDC - 1600 μM/ NHS - 400 μM	Sulfo-EGS (water)	Sulfo-EGS (PBS)
5.972	11.079	34.875	25.772	69.417	17.281	10.965
7.462	10.914	43.573	18.004	69.894	13.047	10.155
7.599	15.049	49.661	20.344	74.070	10.530	14.770
6.976	9.900	33.400	14.069	68.356	12.572	12.174
9.381	17.769	42.187	14.584	71.171	21.557	12.400
8.071		54.807	21.566	79.256		

Table C11. One Way Anova results used for testing of inequality of crosslinkers used when P-CRC was grafted on 10% collagen scaffolds and further washed with 10% SDS-PBS (counts, sums, averages and variances).

Anova: Single Factor SUMMARY				
Groups	**Count**	**Sum**	**Average (ng)**	**Variance (ng)**
NO X-LINKING	6	45.462	7.577	1.290
EDC – 200 μM	5	64.711	12.942	11.144
EDC – 1600 μM	6	258.504	43.084	68.580
EDC - 200 uM/NHS – 50 μM	6	114.338	19.056	19.808
EDC - 1600 μM/NHS - 400 μM	6	432.163	72.027	16.402
Sulfo-EGS (water)	5	74.987	14.997	19.471
Sulfo-EGS (PBS)	5	60.464	12.093	3.074

Table C 12: One Way Anova results obtained for testing of inequality of crosslinkers used when P-CRC was grafted on 10% collagen scaffolds and further washed with 10% SDS-PBS

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	19178	6	3196	154	3.645E-22	2.399
Within Groups	665	32	21			
Total	19844	38				

Section 3: Raw data for P-CRC grafting to 10% collagen scaffolds and further stability evaluation in 10% FBS Solutions.

Table C13: Raw data for P-CRC stock solutions prepared for the experiment involving grafting of peptide with EDC and NHS chemicals and further immersion in 10% FBS solutions

Serum stability study
background cpm 45

50 μM P-CRC in 10 mM phosphate, pH7.5
50μL

Count 1 (cpm)	Count 1 Corrected (cpm)	Count 2 (cpm)	Count 2 Corrected (cpm)	Count 3 (cpm)	Count 3 Corrected (cpm)	Avg. Count (cpm)	Standard Deviation of Counts (cpm)	actual (cpm/mL)	Actual (cpm/mg)
49851	49806	49291	49246	49332	49287	49446	312	988923	1104819

100μL

Count 1 (cpm)	Count 1 Corrected (cpm)	Count 2 (cpm)	Count 2 Corrected (cpm)	Count 3 (cpm)	Count 3 Corrected (cpm)	Avg. Count (cpm)	Standard Deviation of Counts (cpm)	actual (cpm/mL)	Actual (cpm/mg)
97038	96993	97153	97108	98388	98343	97481	748	974812	1089053
								Final Average	1096936

Table C 14: Raw data for P-CRC grafting with EDC and NHS chemicals to 10% collagen scaffolds and further immersion in 10% FBS solution.

No X-Linking		**1096936** (cpm/mg)													
Time (hrs)	**Count 1 (cpm)**	**Count 1 Corrected(cpm)**	**Amount (ng)**	**Count 2(cpm)**	**Count 2 Corrected(cpm)**	**Amount (ng)**	**Count 3(cpm)**	**Count 3 Corrected(cpm)**	**Amount (ng)**	**Avg (ng)**	**Stdev(ng)**	**Stderror (ng)**	**Fraction remaining relative to**	**Error**	**Average (ng/cm^3)**

													0hr sample		
0	13997	13952	12719	14847	14802	13493	15222	15177	13835	13349	572	330	1	0.036	844873
10	1193	1148	1047	1194	1149	1047	1223	1178	1074	1056	16	9	0.080	0.002	66834
24	872	827	754	997	952	868	856	811	739	787	70	41	0.060	0.003	49813
48	694	649	592	814	769	701	711	666	607	633	59	34	0.048	0.003	40071
125	388	343	313	332	287	262	301	256	233	269	40	23	0.020	0.002	17031
220	180	135	123	142	97	88	160	115	105	105	17	10	0.008	0.001	6674
336	89	44	40	96	51	46	74	29	26	38	10	6	0.003	0.000	2375

100 µM – EDC

Time (hrs)	Count 1 (cpm)	Count 1 Corrected(cpm)	Amount (ng)	Count 2(cpm)	Count 2 Corrected(cpm)	Amount (ng)	Count 3(cpm)	Count 3 Corrected(cpm)	Amount (ng)	Avg (ng)	Stdev(ng)	Stderror (ng)	Fraction remaining relative to 0hr sample	Error	Average (ng/cm^3)
0	15067	15022	13694	15534	15489	14120	15215	15170	13829	13881	218	126	1	0.014	878550
10	1743	1698	1548	1893	1848	1685	1988	1943	1771	1668	112	65	0.121	0.005	105559
24	1269	1224	1116	1168	1123	1024	1176	1131	1031	1057	51	30	0.077	0.002	66891
48	905	860	784	1083	1038	946	845	800	729	820	113	65	0.060	0.005	51890
125	710	665	606	508	463	422	562	517	471	500	95	55	0.036	0.004	31619
220	222	177	161	164	119	108	195	150	137	135	26	15	0.010	0.001	8559
336	94	49	44	108	63	57	89	44	40	47	9	5	0.003	0.000	2981

1600 µM – EDC

Time (hrs)	Count 1 (cpm)	Count 1 Corrected(cpm)	Amount (ng)	Count 2(cpm)	Count 2 Corrected(cpm)	Amount (ng)	Count 3(cpm)	Count 3 Corrected(cpm)	Amount (ng)	Avg (ng)	Stdev(ng)	Stderror (ng)	Fraction remaining relative to 0hr sample	Error	Average (ng/cm^3)
0	18560	18515	16878	20829	20784	18947	20269	20224	18436	18087	1078	622	1	0.049	1144759
10	4976	4931	4495	5597	5552	5061	4487	4442	4049	4535	507	293	0.251	0.018	287019
24	5087	5042	4596	5067	5022	4578	5220	5175	4717	4631	76	44	0.256	0.009	293078
48	4037	3992	3639	4186	4141	3775	3888	3843	3503	3639	136	78	0.201	0.008	230331
125	1419	1374	1253	1206	1161	1058				1155	138	97	0.064	0.006	73118
220	387	342	312	636	591	539				425	161	113	0.023	0.006	26916
336	216	171	155	158	113	103	174	129	117	125	27	16	0.007	0.001	7924

100 μM – EDC / 25 μM – NHS

Time (hrs)	Count 1 (cpm)	Count 1 Corrected(cpm)	Amount (ng)	Count 2(cpm)	Count 2 Corrected(cpm)	Amount (ng)	Count 3(cpm)	Count 3 Corrected(cpm)	Amount (ng)	Avg (ng)	Stdev(ng)	Stderror (ng)	Fraction remaining relative to 0hr sample	Error	Average (ng/cm^3)
0	14407	14362	13092	15495	15450	14085	15538	15493	14124	13767	585	337	1	0.034	871328
10	1463	1418	1293	1410	1365	1244	1694	1649	1503	1347	138	79	0.098	0.006	85239
24	1333	1288	1174	1342	1297	1182	1504	1459	1330	1229	88	51	0.089	0.004	77758
48	979	934	851	1308	1263	1151	955	910	829	944	180	104	0.069	0.008	59746
125	418	373	340	609	564	514	427	382	348	401	98	57	0.029	0.004	25349
220	174	129	118	243	198	181	166	121	110	136	39	22	0.010	0.002	8607
336	85	40	36	81	36	32	84	39	35	34	2	1	0.003	0.000	2183

400 μM – EDC / 100 μM – NHS

Time (hrs)	Count 1 (cpm)	Count 1 Corrected(cpm)	Amount (ng)	Count 2(cpm)	Count 2 Corrected(cpm)	Amount (ng)	Count 3(cpm)	Count 3 Corrected(cpm)	Amount (ng)	Avg (ng)	Stdev(ng)	Stderror (ng)	Fraction remaining relative to 0hr sample	Error	Average (ng/cm^3)
0	16041	15996	14582	17120	17075	15566	16318	16273	14835	14994	511	295	1	0.030	949019
10	3257	3212	2928	5602	5557	5066	3984	3939	3590	3862	1094	632	0.257	0.042	244400
24	3747	3702	3375	3863	3818	3481	3906	3861	3520	3458	75	43	0.230	0.006	218887
48	2761	2716	2476	3002	2957	2695	3153	3108	2833	2668	180	104	0.177	0.008	168873
125	1077	1032	940	1278	1233	1124	1657	1612	1470	1178	269	155	0.078	0.010	74556
220	242	197	180	349	304	277	352	307	279	245	57	33	0.016	0.002	15521
336	174	129	117	190	145	132	96	51	46	98	46	26	0.007	0.002	6231

1600 μM - EDC / 400 μM – NHS

Time (hrs)	Count 1 (cpm)	Count 1 Corrected(cpm)	Amount (ng)	Count 2(cpm)	Count 2 Corrected(cpm)	Amount (ng)	Count 3(cpm)	Count 3 Corrected(cpm)	Amount (ng)	Avg (ng)	Stdev(ng)	Stderror (ng)	Fraction remaining relative to 0hr sample	Error	Average (ng/cm^3)
0	19750	19705	17963	18713	18668	17018	19314	19269	17566	17516	474	274	1	0.020	1108602
10	15499	15454	14088	16525	16480	15024	14507	14462	13184	14098	920	531	0.806	0.032	892301
24	12228	12183	11106	13762	13717	12505	13874	13829	12607	12073	839	484	0.690	0.029	764086
48	15020	14975	13651	13600	13555	12357	12646	12601	11487	12498	1089	629	0.715	0.037	791031

125	2765	2720	2479	2516	2471	2253	2632	2587	2358	2363	113	65	0.135	0.004	149582
220	901	856	780	775	730	665	700	655	597	681	92	53	0.039	0.003	43091
336	477	432	394	253	208	190	538	493	449	344	137	79	0.020	0.005	21781

Examples of Calculations :

Calculations for Stock Solutions of Serum Stability samples (calculations for correction of accounts to take into account background radioactivity emission, averages, standard deviations, and standard errors will not be repeated for the three different experiments done in this project as they follow the same formulas).

Calculations for the values obtained at 50μl of the 50 μM P-CRC in 10 mM phosphate (stock solution, refer to Table C13).

Count 1 Corrected = Count 1 – Background cpm = 49851 - 45 = 49806 cpm

Average count = (count 1 corrected + count 2 corrected + count 3 corrected)/3 = (49291 + 49246 + 49287)/3 = 49446 cpm

Standard Deviation = $\sqrt{\left(\frac{1}{N}\sum_{I=1}^{N}\left(CountCorrected - AverageOfCountsCorrected\right)^2\right)}$

Standard deviation = $\sqrt{\left(\frac{1}{3}\left((49806-49446.16)^2+(49245.5-49446.16)^2+(49287-49446.16)^2\right)\right)}$ =312.31 cpm

Actual cpm/ml = $\frac{AverageCpm \times 20}{1ml} = \frac{49446.16 \times 20}{1}$ =988923.33 cpm/ml

(multiplication by 20 is required to bring initial amount used (50ul) to total amount prepared at 1 ml)

Actual cpm/mg= $\frac{actualcpm/ml}{TheoreticalValue\,\mathrm{Pr}\,eparedInSolution(mg/ml)} = \frac{988923.33(cpm/ml)}{0.8951(mg/ml)}$ =1104818.828 cpm/mg

Theoretical Value Prepared in Solution : 50 uM x $10^{-6}\,\frac{\mu mol}{L} \times \frac{1mol}{10^6\,\mu mol}$ x 17902 g/mol =

0.8951 mg/ml

Calculations for No X-linking sample of Serum stability data

Calculations of the percentage of remaining peptides in collagen scaffolds after x hrs in serum. (Calculations will be done only for the No X-linking sample). The calculations for the corrected counts, average and standard deviation will be omitted as they are the same as presented above. Therefore the percentage of P-CRC remaining in collagen scaffolds is :

Average cpm/mg from stock solutions =

$$\frac{1104818.828+1089053.365}{2}=1096936.097 cpm/mg$$

$$\text{Amount (ng)} = \frac{count1corrected}{AverageCpm/mg} = \frac{13952cpm}{1096936.097} = 12719.063ng$$

$$\text{Standard Error at hour 0} = \frac{S\tan dardDeviation}{\sqrt{N}} = \frac{571.943}{\sqrt{3}} = 330.211\,\text{cpm}$$

Average amount remaining at hour 0 = 13349.3 ng

Average amount remaining at hour 10 = 1055.971 ng

$$\text{Percentage remaining at hour 10} = \frac{Amount\,\text{Re}\,mainingAtHour10}{Amount\,\text{Re}\,mainingAtHour0}\times 100 = \frac{1055.971}{13349.3} = 7.98\%$$

Volume of sample used corresponds to the volume of a cylinder as samples were small disks at 0.05 cm thickness.

Volume of sample = Surface Area of Disk x thickness of disk = π x R^2 x d = 3.14 x (0.317 cm)^2 x 0.05 cm=0.0158 cm^3

$$\text{Amount of cpm/cm}^3 = \frac{AverageAmount(ng)}{VolumeOfSample} = \frac{13349ng}{0.0158cm^3} = 844873ng/cm^3$$

All One Way and Two Way Anova results were obtained by use of Microsoft Excel Software.

Statistics for serum stability

Table C 15. Data corresponding to the amount of P-CRC peptide (ng) in each sample inmmersed in 10% FBS solutions. This data was used for statistical Analysis by Two Way Anova with replication (triplicates of each sample are shown for each crosslinker and at every hour studied). Note that due to lack of data, the values for 1600uM-EDC were not taken into account in test.

Time (hrs)	No X-Linking	100 uM - EDC	400 uM - EDC	100 uM - EDC / 25 uM - NHS	400 uM - EDC / 100 uM - NHS	1600 uM - EDC / 400 uM - NHS	1600 uM - EDC
	13835	13694	16232	13092	14582	17963	16878
0hr	12719	14120	15009	14085	15566	17018	18947
	13493	13829	15240	14124	14835	17566	18436
	1074	1548	2293	1293	2928	14088	4495
10hr	1047	1685	2773	1244	5066	15024	5061
	1047	1771	2356	1503	3590	13184	4049
	868	1116	1404	1174	3375	11106	4596
24hr	754	1024	1670	1182	3481	12505	4578
	739	1031	1769	1330	3520	12607	4717
	701	784	1565	851	2476	13651	3639
48hr	592	946	1620	1151	2695	12357	3775
	607	729	1498	829	2833	11487	3503
	262	606	350	340	940	2479	1253
125hr	313	422	339	514	1124	2253	1058
	233	471	485	348	1470	2358	
	88	161	86	118	180	780	312
220hr	123	108	234	181	277	665	539
	105	137	146	110	279	597	
	46	44	23	36	117	394	155
336hr	40	57	32	32	132	190	103
	26	40	30	35	46	449	117

Table C 16: Anova: Two-Factor Anova with Replication of data corresponding to the amount of P-CRC peptide (ng) in each sample inmmersed in 10% FBS solutions.

SUMMARY	100 uM - EDC	400 uM - EDC	100 uM - EDC / 25 uM - NHS	400 uM - EDC / 100 uM - NHS	1600 uM - EDC / 400 uM – NHS	Total
13835						
Count	3	3	3	3	3	15
Sum	41643	46481	41301	44983	52548	226956
Average	13881	15494	13767	14994	17516	15130
Variance	47446	422353	341704	260980	225108	2169813
1074						
Count	3	3	3	3	3	15
Sum	5003	7422	4040	11585	42295	70345
Average	1668	2474	1347	3862	14098	4690
Variance	12634	67964	18952	1197622	846598	24826011
868						
Count	3	3	3	3	3	15
Sum	3171	4843	3686	10375	36218	58292
Average	1057	1614	1229	3458	12073	3886
Variance	2620	35466	7672	5622	703389	18842740
701						
Count	3	3	3	3	3	15
Sum	2460	4683	2832	8005	37495	55474
Average	820	1561	944	2668	12498	3698
Variance	12733	3714	32384	32475	1185990	21382620
262						
Count	3	3	3	3	3	15
Sum	1499	1174	1202	3534	7090	14499
Average	500	391	401	1178	2363	967
Variance	9077	6666	9628	72195	12849	629739
88						
Count	3	3	3	3	3	15
Sum	406	466	408	736	2043	4058
Average	135	155	136	245	681	271
Variance	701	5515	1501	3233	8529	49636
46						
Count	3	3	3	3	3	15
Sum	141	85	103	295	1032	1658
Average	47	28	34	98	344	111
Variance	77	22	4	2078	18668	18250
Total						
Count	21	21	21	21	21	
Sum	54323	65154	53572	79513	178721	
Average	2587	3103	2551	3786	8511	
Variance	22611277	27655872	22296597	24146866	46303960	

Table C 17: Summary of results for Two-Factor Anova with Replication of data corresponding to the amount of P-CRC peptide (ng) in each sample inmmersed in 10% FBS solutions.

ANOVA						
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Sample	2439354263	6	406559044	2535	9.041E-80	2.231
Columns	529926158	4	132481539	826	4.388E-58	2.503
Interaction	409708834	24	17071201	106	8.187E-46	1.674
Within	11228341	70	160405			
Total	3390217596	104				

Appendix D

Tukey Scheffe tests for the MSC culturing data done for days 1, 3, 5, 7, 9, 11 and 13 with the software Origin. The letters A, B, C, D, E, F, G and H correspond respectively to the following treatments: P-CRC, CRC-RGDS, CRC-LQVQ, CRC-RGDS/CRC-LQVQ, Unmodified 10% collagen hydrogel, EDC modified 10% collagen hydrogel, CRC-RGDS/CRC-LQVQ containing only well and control consisting of an empty polystyrene well.

Day 1

[11/21/2008 23:09 "/Data1" (2454791)]

One-Way ANOVA

Summary Statistics

Dataset	N	Mean	SD	SE
Data1_A	6	13.66667	2.42212	0.98883
Data1_B	6	13.16667	4.83391	1.97343
Data1_C	6	10.5	5.1672	2.1095
Data1_D	6	10	1.67332	0.68313
Data1_E	6	9.66667	4.17931	1.7062
Data1_F	6	6.5	3.39116	1.38444
Data1_G	6	3.5	1.51658	0.61914
Data1_H	6	12.5	4.46094	1.82117

Null Hypothesis: The means of all selected datasets are equal
Alternative Hypothesis: The means of one or more selected datasets are different

ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	7	507.312500	72.4732143	5.27558	0.00025
Error	40	549.500000	13.7375000		

At the 0.05 level,
the population means are significantly different.

Means Comparison using Tukey Test

Dataset	Mean	Difference between Means	Simultaneous Confidence Intervals Lower Limit	Upper Limit	Significant at 0.05 Level
Data1_A	13.66667				
Data1_B	13.16667	0.5	-6.34022	7.34022	No
Data1_C	10.5	3.16667	-3.67355	10.00689	No
Data1_D	10	3.66667	-3.17355	10.50689	No
Data1_E	9.66667	4	-2.84022	10.84022	No
Data1_F	6.5	7.16667	0.32645	14.00689	Yes
Data1_G	3.5	10.16667	3.32645	17.00689	Yes
Data1_H	12.5	1.16667	-5.67355	8.00689	No
Data1_B	13.16667				
Data1_C	10.5	2.66667	-4.17355	9.50689	No
Data1_D	10	3.16667	-3.67355	10.00689	No
Data1_E	9.66667	3.5	-3.34022	10.34022	No
Data1_F	6.5	6.66667	-0.17355	13.50689	No
Data1_G	3.5	9.66667	2.82645	16.50689	Yes
Data1_H	12.5	0.66667	-6.17355	7.50689	No
Data1_C	10.5				
Data1_D	10	0.5	-6.34022	7.34022	No
Data1_E	9.66667	0.83333	-6.00689	7.67355	No

Data1_F	6.5	4	-2.84022	10.84022	No
Data1_G	3.5	7	0.15978	13.84022	Yes
Data1_H	12.5	-2	-8.84022	4.84022	No

Data1_D	10				
Data1_E	9.66667	0.33333	-6.50689	7.17355	No
Data1_F	6.5	3.5	-3.34022	10.34022	No
Data1_G	3.5	6.5	-0.34022	13.34022	No
Data1_H	12.5	-2.5	-9.34022	4.34022	No

Data1_E	9.66667				
Data1_F	6.5	3.16667	-3.67355	10.00689	No
Data1_G	3.5	6.16667	-0.67355	13.00689	No
Data1_H	12.5	-2.83333	-9.67355	4.00689	No

Data1_F	6.5				
Data1_G	3.5	3	-3.84022	9.84022	No
Data1_H	12.5	-6	-12.84022	0.84022	No

Data1_G	3.5				
Data1_H	12.5	-9	-15.84022	-2.15978	Yes

Day3

[11/21/2008 23:11 "/Data1" (2454791)]

One-Way ANOVA

Summary Statistics

Dataset	N	Mean	SD	SE
Data1_A	6	11.16667	1.7224	0.70317
Data1_B	6	13.66667	2.80476	1.14504
Data1_C	6	14.83333	4.16733	1.70131
Data1_D	6	13.33333	4.32049	1.76383
Data1_E	6	15.66667	3.32666	1.3581
Data1_F	6	13	3.2249	1.31656
Data1_G	6	23.33333	6.21825	2.53859
Data1_H	6	29	5.93296	2.42212

Null Hypothesis: The means of all selected datasets are equal
Alternative Hypothesis: The means of one or more selected datasets are different

ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	7	1588.00000	226.857143	12.76271	0.00000
Error	40	711.000000	17.7750000		

At the 0.05 level,
the population means are significantly different.

Means Comparison using Tukey Test

Dataset	Mean	Difference between Means	Simultaneous Confidence Intervals Lower Limit	Upper Limit	Significant at 0.05 Level
Data1_A	11.16667				
Data1_B	13.66667	-2.5	-10.28074	5.28074	No
Data1_C	14.83333	-3.66667	-11.44741	4.11408	No
Data1_D	13.33333	-2.16667	-9.94741	5.61408	No
Data1_E	15.66667	-4.5	-12.28074	3.28074	No
Data1_F	13	-1.83333	-9.61408	5.94741	No
Data1_G	23.33333	-12.16667	-19.94741	-4.38592	Yes
Data1_H	29	-17.83333	-25.61408	-10.05259	Yes
Data1_B	13.66667				
Data1_C	14.83333	-1.16667	-8.94741	6.61408	No
Data1_D	13.33333	0.33333	-7.44741	8.11408	No
Data1_E	15.66667	-2	-9.78074	5.78074	No
Data1_F	13	0.66667	-7.11408	8.44741	No
Data1_G	23.33333	-9.66667	-17.44741	-1.88592	Yes
Data1_H	29	-15.33333	-23.11408	-7.55259	Yes
Data1_C	14.83333				
Data1_D	13.33333	1.5	-6.28074	9.28074	No

Data1_E	15.66667	-0.83333	-8.61408	6.94741	No
Data1_F	13	1.83333	-5.94741	9.61408	No
Data1_G	23.33333	-8.5	-16.28074	-0.71926	Yes
Data1_H	29	-14.16667	-21.94741	-6.38592	Yes

Data1_D	13.33333				
Data1_E	15.66667	-2.33333	-10.11408	5.44741	No
Data1_F	13	0.33333	-7.44741	8.11408	No
Data1_G	23.33333	-10	-17.78074	-2.21926	Yes
Data1_H	29	-15.66667	-23.44741	-7.88592	Yes

Data1_E	15.66667				
Data1_F	13	2.66667	-5.11408	10.44741	No
Data1_G	23.33333	-7.66667	-15.44741	0.11408	No
Data1_H	29	-13.33333	-21.11408	-5.55259	Yes

Data1_F	13				
Data1_G	23.33333	-10.33333	-18.11408	-2.55259	Yes
Data1_H	29	-16	-23.78074	-8.21926	Yes

Data1_G	23.33333				
Data1_H	29	-5.66667	-13.44741	2.11408	No

Day5

[11/21/2008 23:13 "/Data1" (2454791)]

One-Way ANOVA

Summary Statistics

Dataset	N	Mean	SD	SE
Data1_A	6	16.83333	3.71035	1.51474
Data1_B	6	19	3.09839	1.26491
Data1_C	6	15	3.52136	1.43759
Data1_D	6	12.83333	3.0605	1.24944
Data1_E	6	10.66667	3.32666	1.3581
Data1_F	6	10.5	2.42899	0.99163
Data1_G	6	16.5	3.08221	1.25831
Data1_H	6	15.5	3.61939	1.47761

Null Hypothesis: The means of all selected datasets are equal
Alternative Hypothesis: The means of one or more selected datasets are different

ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	7	385.979167	55.1398810	5.20802	0.00028
Error	40	423.500000	10.5875000		

At the 0.05 level,
the population means are significantly different.

Means Comparison using Tukey Test

Dataset	Mean	Difference between Means	Simultaneous Confidence Intervals Lower Limit	Upper Limit	Significant at 0.05 Level
Data1_A	16.83333				
Data1_B	19	-2.16667	-8.17167	3.83833	No
Data1_C	15	1.83333	-4.17167	7.83833	No
Data1_D	12.83333	4	-2.005	10.005	No
Data1_E	10.66667	6.16667	0.16167	12.17167	Yes
Data1_F	10.5	6.33333	0.32833	12.33833	Yes
Data1_G	16.5	0.33333	-5.67167	6.33833	No
Data1_H	15.5	1.33333	-4.67167	7.33833	No
Data1_B	19				
Data1_C	15	4	-2.005	10.005	No
Data1_D	12.83333	6.16667	0.16167	12.17167	Yes
Data1_E	10.66667	8.33333	2.32833	14.33833	Yes
Data1_F	10.5	8.5	2.495	14.505	Yes
Data1_G	16.5	2.5	-3.505	8.505	No
Data1_H	15.5	3.5	-2.505	9.505	No
Data1_C	15				
Data1_D	12.83333	2.16667	-3.83833	8.17167	No
Data1_E	10.66667	4.33333	-1.67167	10.33833	No

Data1_F	10.5	4.5	-1.505	10.505	No
Data1_G	16.5	-1.5	-7.505	4.505	No
Data1_H	15.5	-0.5	-6.505	5.505	No

Data1_D 12.83333

Data1_E	10.66667	2.16667	-3.83833	8.17167	No
Data1_F	10.5	2.33333	-3.67167	8.33833	No
Data1_G	16.5	-3.66667	-9.67167	2.33833	No
Data1_H	15.5	-2.66667	-8.67167	3.33833	No

Data1_E 10.66667

Data1_F	10.5	0.16667	-5.83833	6.17167	No
Data1_G	16.5	-5.83333	-11.83833	0.17167	No
Data1_H	15.5	-4.83333	-10.83833	1.17167	No

Data1_F 10.5

Data1_G	16.5	-6	-12.005	0.005	No
Data1_H	15.5	-5	-11.005	1.005	No

Data1_G 16.5

Data1_H	15.5	1	-5.005	7.005	No

Day 7

[11/21/2008 23:14 "/Data1" (2454791)]

One-Way ANOVA

Summary Statistics

Dataset	N	Mean	SD	SE
Data1_A	6	15.83333	1.60208	0.65405
Data1_B	6	18.16667	3.43026	1.4004
Data1_C	6	17.5	4.13521	1.68819
Data1_D	6	19	4	1.63299
Data1_E	6	15.83333	2.48328	1.01379
Data1_F	6	16.16667	1.60208	0.65405
Data1_G	6	15.5	4.1833	1.70783
Data1_H	6	15.66667	3.01109	1.22927

Null Hypothesis: The means of all selected datasets are equal
Alternative Hypothesis: The means of one or more selected datasets are different

ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	7	74.2500000	10.6071429	1.02567	0.42856
Error	40	413.666667	10.3416667		

At the 0.05 level,
the population means are not significantly different.

Means Comparison using Tukey Test

Dataset	Mean	Difference between Means	Simultaneous Confidence Intervals Lower Limit	Upper Limit	Significant at 0.05 Level
Data1_A	15.83333				
Data1_B	18.16667	-2.33333	-8.26821	3.60154	No
Data1_C	17.5	-1.66667	-7.60154	4.26821	No
Data1_D	19	-3.16667	-9.10154	2.76821	No
Data1_E	15.83333	0	-5.93487	5.93487	No
Data1_F	16.16667	-0.33333	-6.26821	5.60154	No
Data1_G	15.5	0.33333	-5.60154	6.26821	No
Data1_H	15.66667	0.16667	-5.76821	6.10154	No
Data1_B	18.16667				
Data1_C	17.5	0.66667	-5.26821	6.60154	No
Data1_D	19	-0.83333	-6.76821	5.10154	No
Data1_E	15.83333	2.33333	-3.60154	8.26821	No
Data1_F	16.16667	2	-3.93487	7.93487	No
Data1_G	15.5	2.66667	-3.26821	8.60154	No
Data1_H	15.66667	2.5	-3.43487	8.43487	No
Data1_C	17.5				
Data1_D	19	-1.5	-7.43487	4.43487	No
Data1_E	15.83333	1.66667	-4.26821	7.60154	No

Data1_F	16.16667	1.33333	-4.60154	7.26821	No
Data1_G	15.5	2	-3.93487	7.93487	No
Data1_H	15.66667	1.83333	-4.10154	7.76821	No

Data1_D	19

Data1_E	15.83333	3.16667	-2.76821	9.10154	No
Data1_F	16.16667	2.83333	-3.10154	8.76821	No
Data1_G	15.5	3.5	-2.43487	9.43487	No
Data1_H	15.66667	3.33333	-2.60154	9.26821	No

Data1_E	15.83333

Data1_F	16.16667	-0.33333	-6.26821	5.60154	No
Data1_G	15.5	0.33333	-5.60154	6.26821	No
Data1_H	15.66667	0.16667	-5.76821	6.10154	No

Data1_F	16.16667

Data1_G	15.5	0.66667	-5.26821	6.60154	No
Data1_H	15.66667	0.5	-5.43487	6.43487	No

Data1_G	15.5

Data1_H	15.66667	-0.16667	-6.10154	5.76821	No

Day 11

[11/21/2008 23:16 "/Data1" (2454791)]

One-Way ANOVA

Summary Statistics

Dataset	N	Mean	SD	SE
Data1_A	6	12.66667	2.50333	1.02198
Data1_B	6	15	2.09762	0.85635
Data1_C	6	13	2	0.8165
Data1_D	6	13	2.28035	0.93095
Data1_E	6	12.66667	3.32666	1.3581
Data1_F	6	12.66667	2.33809	0.95452
Data1_G	6	10.5	2.16795	0.88506
Data1_H	6	17	1.78885	0.7303

Null Hypothesis: The means of all selected datasets are equal
Alternative Hypothesis: The means of one or more selected datasets are different

ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	7	154.812500	22.1160714	3.99387	0.00213
Error	40	221.500000	5.53750000		

At the 0.05 level,
the population means are significantly different.

Means Comparison using Tukey Test

Dataset	Mean	Difference between Means	Simultaneous Confidence Intervals Lower Limit	Upper Limit	Significant at 0.05 Level
Data1_A	12.66667				
Data1_B	15	-2.33333	-6.67617	2.0095	No
Data1_C	13	-0.33333	-4.67617	4.0095	No
Data1_D	13	-0.33333	-4.67617	4.0095	No
Data1_E	12.66667	0	-4.34283	4.34283	No
Data1_F	12.66667	0	-4.34283	4.34283	No
Data1_G	10.5	2.16667	-2.17617	6.5095	No
Data1_H	17	-4.33333	-8.67617	0.0095	No
Data1_B	15				
Data1_C	13	2	-2.34283	6.34283	No
Data1_D	13	2	-2.34283	6.34283	No
Data1_E	12.66667	2.33333	-2.0095	6.67617	No
Data1_F	12.66667	2.33333	-2.0095	6.67617	No
Data1_G	10.5	4.5	0.15717	8.84283	Yes
Data1_H	17	-2	-6.34283	2.34283	No
Data1_C	13				
Data1_D	13	0	-4.34283	4.34283	No
Data1_E	12.66667	0.33333	-4.0095	4.67617	No

Data1_F	12.66667	0.33333	-4.0095	4.67617	No
Data1_G	10.5	2.5	-1.84283	6.84283	No
Data1_H	17	-4	-8.34283	0.34283	No

Data1_D	13

Data1_E	12.66667	0.33333	-4.0095	4.67617	No
Data1_F	12.66667	0.33333	-4.0095	4.67617	No
Data1_G	10.5	2.5	-1.84283	6.84283	No
Data1_H	17	-4	-8.34283	0.34283	No

Data1_E	12.66667

Data1_F	12.66667	0	-4.34283	4.34283	No
Data1_G	10.5	2.16667	-2.17617	6.5095	No
Data1_H	17	-4.33333	-8.67617	0.0095	No

Data1_F	12.66667

Data1_G	10.5	2.16667	-2.17617	6.5095	No
Data1_H	17	-4.33333	-8.67617	0.0095	No

Data1_G	10.5

Data1_H	17	-6.5	-10.84283	-2.15717	Yes

Day 13

[11/21/2008 23:17 "/Data1" (2454791)]

One-Way ANOVA

Summary Statistics

Dataset	N	Mean	SD	SE
Data1_A	6	13.33333	1.96638	0.80277
Data1_B	6	14.66667	2.25093	0.91894
Data1_C	6	12.66667	1.8619	0.76012
Data1_D	6	14	2.19089	0.89443
Data1_E	6	12.66667	3.14113	1.28236
Data1_F	6	14.33333	1.75119	0.71492
Data1_G	6	13	5.32917	2.17562
Data1_H	6	11.83333	1.83485	0.74907

Null Hypothesis: The means of all selected datasets are equal
Alternative Hypothesis: The means of one or more selected datasets are different

ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	7	38.8125000	5.54464286	0.71659	0.65839
Error	40	309.500000	7.73750000		

At the 0.05 level,
the population means are not significantly different.

Means Comparison using Tukey Test

Dataset	Mean	Difference between Means	Simultaneous Confidence Intervals Lower Limit	Upper Limit	Significant at 0.05 Level
Data1_A	13.33333				
Data1_B	14.66667	-1.33333	-6.46687	3.8002	No
Data1_C	12.66667	0.66667	-4.46687	5.8002	No
Data1_D	14	-0.66667	-5.8002	4.46687	No
Data1_E	12.66667	0.66667	-4.46687	5.8002	No
Data1_F	14.33333	-1	-6.13354	4.13354	No
Data1_G	13	0.33333	-4.8002	5.46687	No
Data1_H	11.83333	1.5	-3.63354	6.63354	No
Data1_B	14.66667				
Data1_C	12.66667	2	-3.13354	7.13354	No
Data1_D	14	0.66667	-4.46687	5.8002	No
Data1_E	12.66667	2	-3.13354	7.13354	No
Data1_F	14.33333	0.33333	-4.8002	5.46687	No
Data1_G	13	1.66667	-3.46687	6.8002	No
Data1_H	11.83333	2.83333	-2.3002	7.96687	No
Data1_C	12.66667				
Data1_D	14	-1.33333	-6.46687	3.8002	No
Data1_E	12.66667	0	-5.13354	5.13354	No

Data1_F	14.33333	-1.66667	-6.8002	3.46687	No
Data1_G	13	-0.33333	-5.46687	4.8002	No
Data1_H	11.83333	0.83333	-4.3002	5.96687	No

Data1_D	14

Data1_E	12.66667	1.33333	-3.8002	6.46687	No
Data1_F	14.33333	-0.33333	-5.46687	4.8002	No
Data1_G	13	1	-4.13354	6.13354	No
Data1_H	11.83333	2.16667	-2.96687	7.3002	No

Data1_E	12.66667

Data1_F	14.33333	-1.66667	-6.8002	3.46687	No
Data1_G	13	-0.33333	-5.46687	4.8002	No
Data1_H	11.83333	0.83333	-4.3002	5.96687	No

Data1_F	14.33333

Data1_G	13	1.33333	-3.8002	6.46687	No
Data1_H	11.83333	2.5	-2.63354	7.63354	No

Data1_G	13

Data1_H	11.83333	1.16667	-3.96687	6.3002	No

Day 9

[11/21/2008 23:18 "/Data1" (2454791)]

One-Way ANOVA

Summary Statistics

Dataset	N	Mean	SD	SE
Data1_A	6	17.5	2.88097	1.17615
Data1_B	6	16.16667	3.37145	1.37639
Data1_C	6	14.16667	2.63944	1.07755
Data1_D	6	15	3.74166	1.52753
Data1_E	6	13.33333	2.33809	0.95452
Data1_F	6	9.33333	1.63299	0.66667
Data1_G	6	8.83333	0.75277	0.30732
Data1_H	6	16.33333	4.0332	1.64655

Null Hypothesis: The means of all selected datasets are equal
Alternative Hypothesis: The means of one or more selected datasets are different

ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	7	432.666667	61.8095238	7.53775	0.00001
Error	40	328.000000	8.20000000		

At the 0.05 level,
the population means are significantly different.

Means Comparison using Tukey Test

Dataset	Mean	Difference between Means	Simultaneous Confidence Intervals Lower Limit	Upper Limit	Significant at 0.05 Level
Data1_A	17.5				
Data1_B	16.16667	1.33333	-3.9514	6.61807	No
Data1_C	14.16667	3.33333	-1.9514	8.61807	No
Data1_D	15	2.5	-2.78473	7.78473	No
Data1_E	13.33333	4.16667	-1.11807	9.4514	No
Data1_F	9.33333	8.16667	2.88193	13.4514	Yes
Data1_G	8.83333	8.66667	3.38193	13.9514	Yes
Data1_H	16.33333	1.16667	-4.11807	6.4514	No
Data1_B	16.16667				
Data1_C	14.16667	2	-3.28473	7.28473	No
Data1_D	15	1.16667	-4.11807	6.4514	No
Data1_E	13.33333	2.83333	-2.4514	8.11807	No
Data1_F	9.33333	6.83333	1.5486	12.11807	Yes
Data1_G	8.83333	7.33333	2.0486	12.61807	Yes
Data1_H	16.33333	-0.16667	-5.4514	5.11807	No
Data1_C	14.16667				
Data1_D	15	-0.83333	-6.11807	4.4514	No
Data1_E	13.33333	0.83333	-4.4514	6.11807	No

Data1_F	9.33333	4.83333	-0.4514	10.11807	No
Data1_G	8.83333	5.33333	0.0486	10.61807	Yes
Data1_H	16.33333	-2.16667	-7.4514	3.11807	No

Data1_D	15

Data1_E	13.33333	1.66667	-3.61807	6.9514	No
Data1_F	9.33333	5.66667	0.38193	10.9514	Yes
Data1_G	8.83333	6.16667	0.88193	11.4514	Yes
Data1_H	16.33333	-1.33333	-6.61807	3.9514	No

Data1_E	13.33333

Data1_F	9.33333	4	-1.28473	9.28473	No
Data1_G	8.83333	4.5	-0.78473	9.78473	No
Data1_H	16.33333	-3	-8.28473	2.28473	No

Data1_F	9.33333

Data1_G	8.83333	0.5	-4.78473	5.78473	No
Data1_H	16.33333	-7	-12.28473	-1.71527	Yes

Data1_G	8.83333

Data1_H	16.33333	-7.5	-12.78473	-2.21527	Yes

Appendix E

Langmuir and Freundlich plots of the P-CRC data loaded to 10% collagen scaffolds.

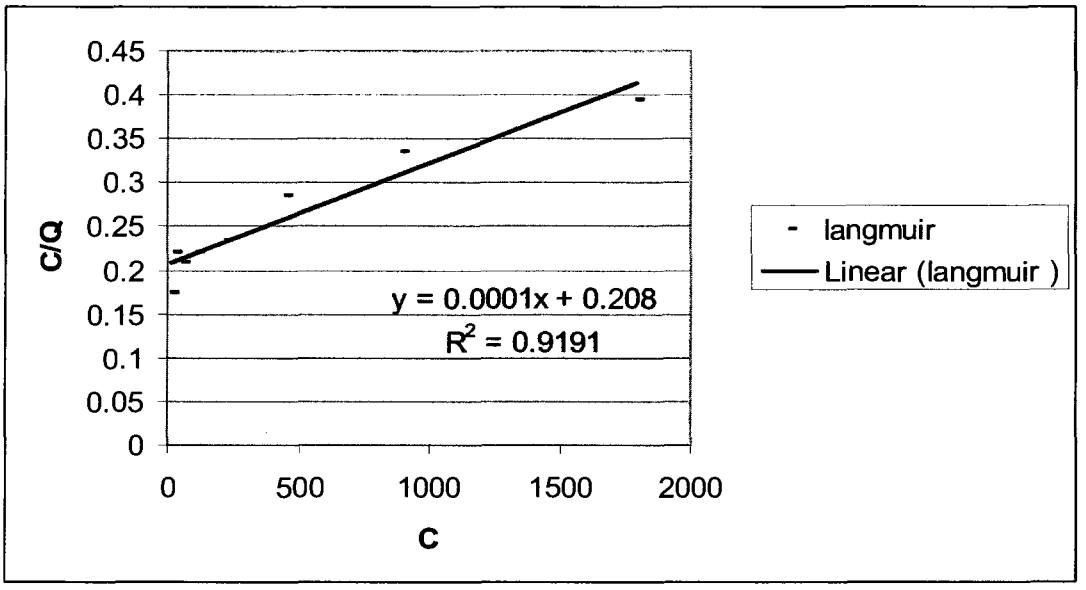

Figure 17: Langmuir plot of the P-CRC loading data to 10% collagen scaffolds obtained when ^{125}I radiolabelled P-CRC was loaded to the collagen scaffolds (without crosslinkers) and radioactivity emissions were then measured.

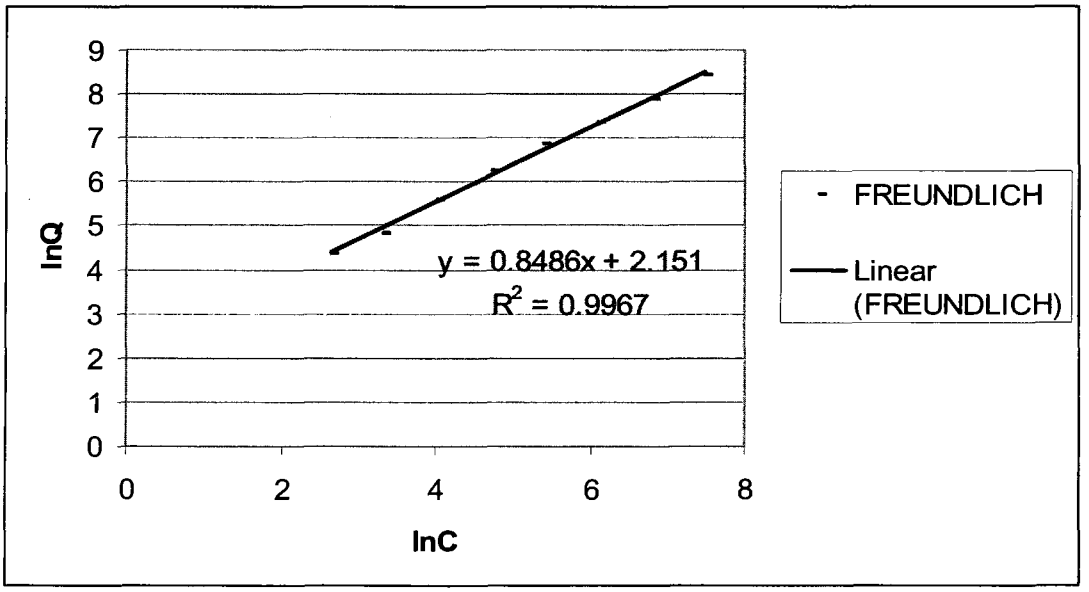

Figure 18: Freundlich plot of the P-CRC loading data to 10% collagen scaffolds obtained when ^{125}I radiolabelled P-CRC was loaded to the collagen scaffolds (without crosslinkers) and radioactivity emissions were then measured.

Calculation examples for Figure 18 since only the values from this plot were used in the thesis.

To obtain the Freundlich parameters, the equation obtained in Figure 18 was used.
Hence:
$Y=0.8486X + 2.151$

From Equation 2, the adsorption constant k_F and the affinity coefficient n can be obtained as follows:

$\text{Ln } k_F = 2.151$

a. $k_F = \exp(2.151)$
b. $k_F = 8.59$

$n = 0.8486$